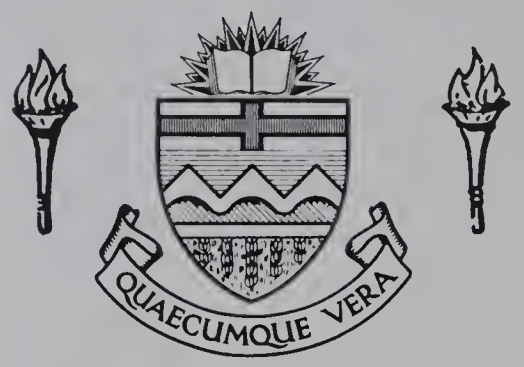

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REPAIR OF γ-RAY INDUCED DNA STRAND BREAKS IN RADIATION SENSITIVE MUTANTS OF YEAST

by

Michael R. A. Mowat

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Repair of gamma-ray induced DNA Strand Breaks in Radiation Sensitive Mutants of Yeast, submitted by Michael R.A. Mowat.

ABSTRACT

The repair of γ-ray induced single and double-strand breaks in DNA of wild type and radiation sensitive mutants (*rad51-1* and *rad18-2*) of yeast was studied using rate-zonal sedimentation.

Wild type diploid yeast were found to repair both single and double-strand breaks. Diploid strains homozygous for *rad51-1* were unable to repair double-strand breaks after 30 krads of γ-irradiation. These strains were able to repair single-strand breaks as well as apurinic sites in the DNA. A low molecular weight component was found in gradients of *rad51-1* strains. This component is probably not DNA since purification of DNA from *rad51-1* strains by CsCl isopycnic centrifugation results in loss of this component giving similar profiles to wild type DNA.

Strains homozygous for *rad18-2* have an ability to repair single-strand breaks and appear to repair double-strand breaks. Upon further incubation the DNA in *rad18-2* strains becomes broken up giving both single and double-strand breaks. This was not seen in wild type cells. From these findings, the possible roles the *RAD51* and *RAD18* gene products play in repair in yeast is discussed.

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INTRODUCTION

At the present time, the mechanisms for repair of ionizing radiation damage to DNA in any organism have not been worked out in full detail. This is due to the many classes of lesion introduced into DNA by ionizing radiation and the several repair mechanisms a cell may utilize (for reviews see Hutterman *et al.*, 1978; Hanawalt and Setlow, 1975; Lehman and Bridges, 1977). This study was undertaken to increase the understanding of repair of γ-ray induced strand breaks in DNA of yeast cells.

Chemical Changes in γ-Irradiated DNA

One of the major types of damage to DNA caused by gamma irradiation is base alterations (Hutterman *et al.*, 1978). The pyrimidine bases in DNA are twice as sensitive as purines to chemical changes after γ-irradiation. The $OH^{\cdot}$ radicals, produced by radiolysis of water, attack predominantly at the 5-6 double bond in pyrimidines. The pyrimidine peroxide produced is very labile which results in more stable glycols (i.e., 5,6-dihydroxy -5,6 dihydrothymine) or opening of the pyrimidine ring between the number 5 and 6 carbons (Teoule *et al.*, 1977). The opened ring is also an unstable structure and results in the formation of 5-hydroxy-5-methylhydantoin or through the loss of carbon dioxide or pyruvamide the formation of formamide, urea or formyl urea in place of the base. These latter structures result in an apyrimidinic site. Bases can also be lost by cleavage of the N-glycosidic bond and liberation of the base resulting in an apurinic or apyrimidinic site (APsites).

The other major damage to DNA induced by γ-irradiation is strand breakage. Damage to the deoxyribose sugar can lead to strand breaks

in a direct fashion by causing release of the adjoining monophosphate group. Direct breaks also occur by breaks at the phosphodiester bond resulting in PO_4 end groups on one side of the break and OH groups on the other side. DNA double-strand breaks can be due to direct breakage of both chains or close proximity of single-strand breaks (Hutterman *et al.*, 1978). AP sites in DNA, formed by γ-irradiation, can lead spontaneously to strand breaks at a slow rate under physiological conditions. Breaks at AP sites can also occur at a much faster rate under alkaline conditions (Lindahl and Andersson, 1972). AP sites can also be substrate for endonucleases which will lead to strand breakage (to be discussed).

Methyl methanesulfonate (MMS) is referred to as a "radiomimetic" chemical because of the large number of DNA strand breaks formed after exposure to MMS (Strauss *et al.*, 1975). MMS alkylates DNA bases with 7-methyl-guanine being the major product. Methylated purines such as 7-methyl-guanine and 3-methyl-adenine are unstable and are released spontaneously, forming AP sites in the DNA. In addition, phosphotriesters are formed on the backbone phosphates, but their biological consequences are not known at this time.

Mechanisms of Repair of Damaged DNA

The molecular biology of repair is best understood in *Escherichia coli*, so the following discussion will concentrate on that organism.

Single-strand Break Repair

The first demonstration of repair of single-strand breaks in *E. coli* after X-rays was by McGrath and Williams (1966). They were also able to demonstrate that the first radiation sensitive strain of *Escherichia coli* to be isolated (B_{s-1}) could not repair X-ray induced single-strand breaks. As mentioned earlier, DNA single-strand breaks can have many

origins and chemical end groups. Hence, any repair of single-strand breaks seen would be due to an array of repair processes.

Three mechanisms are now known by which *E. coli* repairs strand breaks, each distinguished either by its rapidity or by its growth medium requirements. The first, an ultrafast repair system, which can occur during irradiation, is independent of DNA polymerase I (*polA*) and is presumably mediated by the ligase enzyme (Town *et al.*, 1972). The second, a rapid repair system, occurs in buffer and uses polymerase I and ligase (*lig*) (Youngs and Smith, 1976), with the result that strains defective in the *polA* or *lig* gene products are hypersensitive to X-rays (Bonura *et al.*, 1975a; Dean and Pauling, 1970). When polymerase I is absent because of mutation, cells in buffer with a functional polymerase III (*polC*) can handle some of the breaks normally repaired by polymerase I (Hamelin *et al.*, 1976). DNA polymerase III has been shown to be essential for the third process for the repair of single-strand breaks. This system needs a medium that allows growth and also needs the function of the *lexA*, *recB*, *recC* (exonuclease V) and *recA* gene products (Hamelin *et al.*, 1976; Sedgwick and Bridges, 1972; Kapp and Smith, 1970). Recently, the *recA* gene product has been purified and found to catalyze the formation of duplex DNA from complementary single strands (Weinstock *et al.*, 1979). These authors postulate that the *recA* protein functions by catalysing the annealing of a complementary single-strand region from one DNA duplex to the single-strand region of a homologous duplex. This indicates that a recombination event is needed for some single-strand break repair.

Double-strand Break Repair

The importance of DNA double-strand breaks to cell survival after X-irradiation can be seen from the findings of Bonura *et al.* (1975b).

They found that X-irradiation resulted in 1.3-1.4 double-strand breaks per lethal event in wild type *E. coli* cells. Leenhouts and Chadwick (1978) have shown that the kinetics of double-strand break induction follows the same kinetics as cell death and somatic mutation with ionizing radiation. It therefore appears that approximately one unrepaired double-strand break is lethal to a cell.

In the study by Bonura *et al.* (1975b), they were unable to demonstrate repair of double-strand breaks. Recently, Krasin and Hutchinson (1977) have found repair of double-strand breaks in *E. coli*. This repair is dependent upon the presence of more than one homologous genome within the cell and upon the *recA* gene product. Cells grown on medium with aspartate as the carbon source, containing an average of 1.3 genomes per cell, were unable to repair double-strand breaks. Cells grown on D-glucose containing 4 to 5 genomes per cell, showed repair of double-strand breaks. These results suggest that the repair of DNA double-strand breaks involves recombination with a homologous DNA double helix.

A model for the repair of double-strand breaks, involving hybrid DNA formation and gene conversion, was proposed by Resnick (1976). Using this model, Leenhouts and Chadwick (1978) have proposed a mechanism to explain chromosomal aberrations in which a DNA double-strand break is the initial event. They have also shown the kinetics of double-strand break induction follows the same kinetics as cell death and somatic mutation with ionizing radiation.

Repair of Base Damage in DNA

One method of repairing base damage in DNA is excision repair (for review see Grossman, 1975). This method of repair is the major way *E. coli* cells remove U.V. induced pyrimidine dimers. The scheme is

basically as follows: The *uvrA* gene product, a dimer specific endonuclease, hydrolyes the phosphodiester bond next to the dimer. Then the dimer containing strand is removed for a distance of about 16 nucleotides. The resulting gap is then filled in by DNA polymerase I (*polA*) using the opposite strand as the template and the terminal 5'phosphoryl group is then sealed to the adjacent (unexcised) 3'OH group. Base damage of the 5,6-dihydroxy-5,6-dihydrothymine type, induced by γ-irradiation, is removed in a similar manner to UV dimer excision repair with the exception that the lesion specific endonuclease is different (Hariharan *et al.*, 1975). Gates and Linn (1977) may have found an endonuclease specific to 5,6-dihydroxy-dihydrothymine in *E. coli*. They call it endonuclease III.

Another method of removal of base damage which differs from excision repair is called base excision. This is accomplished by an enzymatic removal of the damaged base by breakage of the N-glycosidic bond without breakage of the phosphodiester backbone (Gossard and Verly, 1978). This type of repair is used on alkylated, γ-irradiated and uracil containing DNA. Two N-glycosylases which excise altered bases have been found in *E. coli*. One is endonuclease II which removes 3-methyladenine and 0-6-methylguanine and some unspecified γ-ray induced base damage (Kirtikar *et al.*, 1976). An N-glycosylase which removes uracil from DNA has been found (Da Roza *et al.*, 1977). Mutants (*ung*$^-$) defective in this enzyme have been isolated (Duncan *et al.*, 1978). After removal of the base, the resulting AP site is subject to attack by AP endonucleases which cut the DNA backbone near the AP site. In *E. coli* two such enzymes have been found (Verly and Rassort, 1975; Ljungquist, 1977). One of these endonucleases is associated with the *xthA* gene and cells mutant for this gene are slightly more sensitive

to MMS and γ-rays than are wild type cells (Yajko and Weiss, 1975). After the AP endonuclease step the nick produced is then subjected to exonuclease III activity. The gap is then filled in by DNA polymerase I and sealed by ligase (Gossard and Verly, 1978).

Post-Replication Repair

When base damage to DNA is not removed before the onset of replication, post replication gaps of about 500 to 1000 nucleotides can form where the polymerase is stopped by the damage, for example at a U.V. induced pyrimidine dimer (Howard-Flanders *et al.*, 1968; Howard-Flanders, 1975 for review). The spacing between the gaps is approximately equal to that between pyrimidine dimers. The dimers are not repaired by excision since there is no complementary strand opposite the dimer to act as template during resynthesis. In wild type *E. coli* cells, after further incubation, these post-replication gaps are repaired (Howard-Flanders *et al.*, 1968).

There appear to be two ways of repairing this type of damage. One pathway is dependent on the *recA* gene product and leads to recombination. One possible model for gap repair involving a recombinational event between the damaged DNA and an undamaged sister duplex or homologous genome is as follows: After replication on a damaged template, the gaps opposite the dimer in the daughter strand can be filled with the undamaged strand of the sister duplex by single-strand invasion. The gap left by the invading strand can be filled in by using the sister strand as template. Finalization of the exchange will result in joining of segments of parental and daughter DNA. If during recombination an isomerization event takes place the dimer may be exchanged into the daughter strand. This mechanism allows for a template upon which excision repair can take place or in the event of

no excision repair (i.e., *uvr* mutants) the dimers can be gradually diluted out by distribution between daughter and parental DNA molecules synthesized after irradiation (Ganesan, 1974).

Another process which the cell uses to repair post-replication gaps is dependent on both *lexA* and *recA* gene products. This process is largely mutagenic. The exact mechanism of this "error prone" repair is not known. However, work by Villani *et al.* (1978) suggests that the ability to replicate past a dimer and fill the gap may be due to the cell's ability to induce a modification in the 5'→3' exonuclease activity of the DNA polymerase. The exonuclease activity is implicated in a role in proof-reading of newly replicated DNA (Brutlag and Kornberg, 1972). It is believed that the proof-reading activity of the polymerase is the reason why the polymerase stops at a dimer. The DNA polymerase will incorporate a nucleotide opposite a dimer and then remove it by the 5'→3' exonuclease due to nonpairing with the dimer. The polymerase will idle at the dimer with a turnover of nucleotide triphosphates and release of nucleotide monophosphates. Inhibition of the exonuclease activity allows replication past the dimer with an increased likelihood of inserting the wrong nucleotide opposite the dimer (or other base damage). This allows increased survival, despite the increase in mutation, by decreasing the number of post-replication gaps (Villani *et al.*, 1978).

Genetics of Repair in *Saccharomyces cerevisiae*

The molecular biology of repair in yeast is not as far advanced as in *E. coli*. However, knowledge of the genetics of repair in yeast is quite extensive. The work on *E. coli* repair gives useful working models for interpreting experiments with radiation sensitive mutants in yeast.

At the present time, there are 81 known genetic loci which effect sensitivity to U.V. or X-rays (Haynes *et al.*, 1979; Catcheside, 1974 for review). Generally, genetic loci that confer radiation sensitivity when mutant are given the symbol *rad*. Only one enzyme defective function has been associated with a radiation sensitive mutant; strains carrying the *cdc9* mutation have been shown defective in ligase activity (Johnston and Nasmyth, 1978). The yeast endonuclease α, which acts on denatured DNA, may be the gene product of the *RAD1* locus, since a *rad1-1* strain has half the specific activity of the wild type (Bryant and Haynes, 1978).

Repair Pathways in Yeast

A large number of *rad* strains can be grouped into three categories based on sensitivity or resistance to mutagens. Members of the first group are sensitive to U.V. and nitrogen mustard (HN_2), but show wild type levels of sensitivity to X-rays. Strains placed in the second group are sensitive to X-rays, MMS, U.V. and HN_2. The third group contains mutants sensitive to X-rays and MMS but only slight sensitivity to U.V. compared to wild type cells (Brendel *et al.*, 1970). Another criterion for grouping of genes controlling radiation sensitivity is by the interaction of double mutant strains with respect to survival after irradiation (Brendel and Haynes, 1973; Game and Cox, 1973; Cox and Game, 1974; Game and Mortimer, 1974). If, in a double mutant strain, an epistatic interaction is seen, that is, the strain is as sensitive as the most sensitive single mutant, one can infer that the genes control steps in the same pathway. A synergistic interaction is said to occur when the double mutant strain shows sensitivity greater than the sum of the single mutant strains. This is interpreted to mean that the two gene products function on two pathways and are competing for the same lesion in the DNA. By these tests, with some supporting biochemical data, three major pathways

(epistasis groups) have been described in yeast, known respectively as the *RAD3*, *RAD18* and *RAD52* pathways. These pathways largely correspond with the chemical mutagen sensitivity spectra (Brendle *et al.*, 1970) but some chemical mutagens such as psoralen cause damage such that it will be repaired by using steps from different pathways to repair the lesion (Jachymczyk, pers. comm., to be discussed).

The *RAD3* Pathway

Mutants that belong to this pathway are sensitive to U.V. and show wild type levels of resistance to X-rays. The following mutants have been placed in this pathway: *rad1*, *rad2*, *rad3*, *rad4*, *rad10*, *rad16*, *rad22* (Nakai and Matsumoto, 1967; Game and Cox, 1972; Brendel and Haynes, 1973; Lawrence and Christensen, 1976; Prakash, 1977b). Of this group, strains carrying one of the *rad1*, *rad2*, *rad3*, *rad4*, *rad10*, and *rad16* mutations have been shown biochemically to be defective in excision of U.V. induced pyrimidine dimers (Unrau *et al.*, 1971; Waters and Moustacchi, 1974; Resnick and Setlow, 1972; Prakash 1975, 1977a,b; Reynolds, 1978).

Strains carrying mutations in loci belonging to this pathway show increased U.V.-induced mutation and recombination frequencies compared to wild type cells. Enhanced U.V.-induced recombination has been shown in diploids homozygous for mutations of the following loci: *rad1*, *rad2*, *rad3* and *rad4* (Snow, 1978; Hunnable and Cox, 1971; Kowalski and Laskowski, 1975). The increase in induced mutagenesis with U.V. over wild type cells has been seen in the above strains, as well as strains carrying mutations in *rad10* and *rad22* (Moustacchi, 1969; Resnick, 1969; Averbeck *et al.*, 1970; Cox and Game, 1974; Eckart *et al.*, 1974; Lawrence and Christensen, 1976). Mutants of *rad1*, *rad2*, *rad3* and *rad4* have been tested for enhancement of spontaneous mutability. Only a *rad3* mutant showed enhanced spontaneous mutability

over the wild type strain (Brychcy and von Borstel, 1977). These increases in recombination and mutation can be explained by the pathway theory. Blockage of the excision pathway through mutation are postulated to result in an increase in the number of lesions that must be processed through other pathways. These other processes repair lesions through either a mutagenic or a recombinagenic mechanism (Game and Cox, 1973; Hastings *et al.*, 1976).

RAD18 Pathway

Mutants that are members of the *rad18* epistasis group show increased sensitivity to U.V., X-rays and several chemical mutagens (Snow, 1967; Cox and Parry, 1968; Resnick, 1969; Lemontt, 1971, Brendel and Haynes, 1973; Prakash, 1974, 1976). Mutant genes that have been identified with this system are: *rad6*, *rad9*, *rad15*, *rad18*, *rad8*, *rev1*, *rev2* and *rev3* (Lawrence and Christensen, 1976). The other major phenotype of these mutants is their reduction in U.V., X-ray and chemically induced mutability (Lawrence and Christensen, 1976; Lemontt, 1971; Prakash, 1974, 1976; McKee and Lawrence, 1978). However, this reduction in induced mutability is dependent upon the locus tested and the mutagen used. The *RAD6* gene product appears to be necessary for all types of mutagenesis. For chemical mutagenesis, *rad6*, *rad9* and *rad15* mutations decrease reversions compared to wild type cells. Mutants in *rad6*, *rad8* and *rev3* block U.V. induced mutagenesis. The other *rad* mutants lower induced mutagenesis depending on the test system and mutagen used. One interesting finding concerned with nitrous acid (HNO_2) mutagenesis is that mutants of *rad18* show an increase in reversion compared to wild type strains, whereas, mutants of *rad6*, *rad9* and *rad15* show a decrease (Prakash, 1976).

Other pleiotropic effects of mutants in this pathway have been found. Strains mutant for *rad6* and *rad18* have been found to be sensitive

to the anti-folate drug trimethoprim (Game *et al.*, 1975). The *RAD6* gene product is also necessary for sporulation, induced recombination and recombination during meiosis (Game and Mortimer, 1974; Game *et al.*, 1978; Cox and Game, 1974). Strains mutant in *rad6* have also been shown to be defective in the repair of double-strand breaks in DNA after treatment with MMS (Chlebowicz and Jachymczyk, 1979). Strains carrying either *rad18* or *rad6* mutations show elevated spontaneous mutation rates compared to wild type strains (von Borstel *et al.*, 1971; Hastings *et al.*, 1976). Diploids homozygous for *rad18-3* showed enhanced frequencies of spontaneous mitotic recombination (Borman and Roman, 1976).

The *RAD52* Pathway

The third group of genes is mainly involved in the control of recovery from X-ray damage but only slightly with recovery from U.V. damage. Based on epistatic interactions in X-ray survival of double mutant strains the following genes are involved in this pathway: *RAD50, RAD51, RAD52, RAD54*. The genes *RAD53, RAD55, RAD56* and *RAD57*, when mutant, share phenotypes with mutant strains in this pathway but have not been tested for epistatic interactions (Game and Mortimer, 1974). Game and Mortimer also found *RAD6* to be in this group based on epistatic interaction of mutant strains tested for survival after X-irradiation. *RAD6* had been previously grouped with genes controlling the *RAD18* pathway based on U.V. survival. The significance of this will be discussed later. Both diploid and haploid strains carrying the *rad52-1* mutation are unable to repair X-ray induced double-strand breaks (Resnick and Martin, 1976; Ho, 1975a).

Pleiotropic effects of mutant strains from this group primarily involve recombination related phenomena. Homozygous *rad52* and *rad51* strains are deficient in induced intragenic recombination (Resnick, 1975;

Saeki *et al.*, 1974; Morrison, 1978). Diploids homozygous for *rad50*, *rad51*, *rad52* and *rad57* all show reduced recombination during meiosis (Game *et al.*, 1978; Prakash and Prakash, 1978; Morrison, 1978). This lack of recombination during meiosis may be related to the reduction in sporulation and spore viability seen in diploids homozygous for any one of the *rads* 50-57 (Game and Mortimer, 1974).

Strains carrying mutations in *RAD51* or *RAD52* have been shown to have increased spontaneous mutation rates (von Borstel *et al.*, 1971, Hastings *et al.*, 1976).

Game and Mortimer (1974) found that a mutant haploid containing *rad6-1* and *rad18-1* showed a sensitivity to X-rays greater than the sum of the single mutants. From previous arguments this would indicate these genes control steps in two different pathways for repair of X-ray damage. It was also reported that a septuple mutant containing *rad50-1*, *rad51-1*, *rad52-1*, *rad53-1*, *rad54-1*, *rad6-1* and *rad18-1* was no more sensitive than the *rad18-1*,*rad6-1* double mutant. This implies no further repair processes were blocked by addition of these mutant genes. The fact that *rad6* mutants share similar phenotypes with other mutant genes in the *rad52* epistasis group (for example, effects on recombination and sporulation) is consistant with the *RAD6* gene product being involved in the same repair process. It therefore seems that the *RAD6* gene product controls a step in the *RAD52* pathway involved in repairing X-ray induced damage (but not in the *RAD18* pathway for repairing X-ray damage) and a step in the *RAD18* pathway for repairing U.V. induced damage.

MMS Sensitive Mutants (*mms*)

Prakash and Prakash (1977a) have isolated MMS sensitive mutants (*mms*) that are allelic to *rad6*, *rad55*, *rad57*, *rad1* and *rad4* and four alleles of *rad52* as well as 16 other complementation groups not allelic to the

known *rad* loci. The surprising finding of some MMS sensitive mutants being allelic to *rad1* and *rad4* which are involved in dimer excision suggest that the same enzymes may be involved in dimer excision and removal of alkylation products. The remaining mutants they found fall into four phenotypic classes: mutants sensitive to MMS only, mutants sensitive to MMS and U.V., mutants sensitive to U.V., X-rays and MMS and finally mutants sensitive to X-rays and MMS. With the exception of the group sensitive only to MMS, the three other classes may be genes involved in the three major repair pathways as defined by radiation. The mutants sensitive to only MMS may represent genes controlling a repair pathway involved in MMS damage not previously described (Prakash and Prakash, 1977a).

Purpose

By studying mutants in the genes that control steps in the two major X-ray repair pathways it was hoped to gain some insight as to their functions. Since, the role of these two pathways in recombination and mutation has been firmly established, knowledge of the function of genes that control steps in these pathways would aid in understanding the mechanisms of recombination and mutation. At the outset of this study, no biochemical evidence as to the role the *RAD18* system in repair of ionizing radiation damage was known. The function of the *RAD52* gene in the repair of double-strand breaks was known, but no evidence had been presented to its ability to repair single-strand breaks had been presented. Also, no other members of the *rad52* epistasis group had been tested for repair of strand breaks.

The approach taken to gain this understanding was to study the ability of *rad18-2* and *rad51-1* mutant strains to repair single and double-strand breaks in their DNA after γ-irradiation. To measure this repair,

the technique of sucrose gradient centrifugation of DNA was used. This technique can also give some information as to the repair of base damage to DNA as well.

MATERIALS AND METHODS

Strains

The genotypes and sources of haploid *Saccharomyces cerevisiae* strains used in this study are given in Table 1. The abbreviations *ura4*, *ade2*, *lys1*, *met10*, *his1*, *trp5*, *arg6*, *leu2* and *hom3* refer to recessive alleles which result in nutritional requirements for uracil, adenine, lysine, methionine, histidine, tryptophane, arginine, leucine and threonine; *a* and α are two alleles of the mating type locus; *cry1* and *can1* are loci that allow resistance to crytopleurine and canavanine sulphate respectively; *SUP6* is a dominant tRNA suppressor locus that removes the nutritional requirement in strains carrying the following mutant genes, *ade2-1; trp5-48; lys1-1;* and removes the resistance to canavanine sulphate caused by the mutation *can1-100*. The *rad* mutants exhibit increased sensitivity to ultraviolet radiation and ionizing radiation. The *rad18-2* (*uvs1-1*) allele was originally isolated by Resnick (1969) and *rad51-1* (*Xs1*) was isolated by Nakai and Matsumato (1967); *mut5-1* was isolated on the basis of its increased spontaneous mutation rate compared to wild type cells (von Borstel *et al.*, 1973) and is an allele of the *RAD51* gene (Morrison, 1978); A *cdc* mutation confers a temperature sensitive cell cycle blockage.

The pedigree of strains constructed for use during this study can be seen in appendix 1. The source of diploids used in this study are given in table 2 and the genotypes of diploid strains analysed by sucrose gradient centrifugation are given in table 3.

Media

YEPD: 1% Bacto-yeast extract, 2% Bacto-peptone, 2% D-glucose in distilled water.

Table 1

Genotypes and source of parental strains employed in this study

Strain	Genotype	Source or Reference
A 14-33:	*ade2-1, lys1-1, trp5-48, can1-100 his2, cdc14*	Dicaprio(1976)
A 14-137	α *ade2-1, lys1-1, trp5-48, can1-100 Sup6-1, met10-4, leu2, ura4-11*	"
A 3-3	*a ade2-1, trp5-48, lys1-1*	"
Z2367 -15C	α *his1-315, arg6, ade1*	Savage
XV185-14C	*a trp5-48, arg4-17, his1-7, lys1-1, ade2-1, hom3-10*	von Borstel
A 1-36D (36DrI)	α *trp5-48, arg4-17, lys1-1, hom3-10*	DiCaprio (1976)
X2180-1A	*a*	Yeast Genetic Stock Centre
Z2367-1B	*a arg6, hom3-10, ura3*	Savage
A 011-32C	α *ura4-11, trp5-48, lys1-1, ade2-1, can1-100, Sup6-1*	DiCaprio (1976)
A 14-8A	*leu2, trp5-48, lys1-1, ade2-1, can1-100, his2, cdc14, Sup6-1, met10-4*	"
A 20-10	*ade2-1, lys1-1, trp5-48, can1-100, sup6-1-10, his2, cdc14, leu2*	"
A 516-2A	*a ura4-11, ade2-1, trp5-48, lys1-1, can1-100, Sup6-1, met10-4, rad51-1*	"
KF 179-10C	*a* cry^R, *ura3, hom3-10, his1-1, mut5, lys1-1, trp5-48, ade2-1*	Morrison (1978)
KF 179-11C	α *ura3, his1-315, arg6, mut5, lys1-1, trp5-48, ade2-1*	"
KF 179-17A	α *ura3, his1-315, arg6, lys1-1, trp5-48*	"
KF 179-16A	*a* cry^R, *ura3, hom3-10, his1-1, lys1-1, trp5-48, ade2-1*	"

Table 1 (cont'd)

Strain	Genotype	Source
Y 011-6C	*ade?, his?, leu1-12, lys1-1, rad18-2*	Quah
Y 011-1C	*lys1-1, his?, ade?, rad18-2*	"
LA 10-35B	α *trp5-48, lys1-1, ade2-1*	see appendix 1
LA 11-3A	*a trp5-48, lys1-1, ade2-1, cdc14*	"
LA 12-19C	α *cdc14, his1-315, arg6, lys1-1, ade2-1, trp5-48*	"
LA 13-3A	α *his1-7, trp5-48, arg4-17, lys1-1, hom3-10*	"
LA 14-17B	α *his1-7, hom3-10*	"
LA 15-18C	α *ura3, his1-7*	"
LA 16-30B	*ura3, trp5-48, lys1-1, ade2-1, his1-7*	"
LA 17-39B(L139)	*leu2, ura3, trp5-48, lys1-1, ade2-1, can1-100, Sup6-1, met10-4*	"
LA 9-3B(L138)	*leu2, ade2-1, lys1-1, trp5-48, can1-100, Sup6-1, met10-4*	"
LA 18-11A	*leu2, ade2-1, lys1-1, trp5-48, can1-100, cdc14, Sup6-1, met10-4*	"
LA 19-1A(L36)	α *leu2, ade2-1, lys1-1, trp5-48, can1-100, his1-315, cdc14, Sup6-1, met10-4*	"
LA 20-3B(L140)	*a ura3, ade2-1, lys1-1, trp5-48, can1-100, his1-7, sup6-1-10, ura?*	"
LA 21-19A(L141)	*a ura4-1, trp5-48, lys1-1, ade2-1, can1-100, his1-7, sup6-1-10*	"
LA 1-22D	α *ade2-1, trp5-48, lys1-1, Sup6-1, met10-4, rad51-1*	"
LA 1-106C LA 1-83B	α *leu2, trp5-48, lys1-1, ade2-1, can1-100, his1-315, Sup6-1, cdc14, met10-4, rad51-1*	"
LA 2-75D	*a ura4-11, ade2-1, lys1-1, trp5-48, sup6-1-10, his1-7, rad51-1, can1-100*	"

TABLE 2

The origin of diploid strains used in this study

Diploid	Haploid parents
LA 1	L 36 X A 516-2A
LA 2	L 140 X LA 1-22D
LA 3	L 141 X L 36
LA 4	Y011-1C X Y011 6C
LA 5	LA 1-83B X LA 2-75D
LA 6	KF 179-10C X KF 179-11C
LA 7	LA 1-106C X LA 2-75D
LA 9	A 14-33 X A 14-137
LA 10	A 14-137 X A 3-3
LA 11	A 14-33 X LA 10-35B
LA 12	LA 11-3A X Z 2367-15C
LA 13	XV 185-14C X 36DrI
LA 14	X 2180-1A X LA 13-3A
LA 15	LA 14-17B X Z 2367-1B
LA 16	L 138 X LA 15-41C
LA 17	L 138 X LA 16-30B
LA 18	A 011-32C X A 14-8A
LA 19	LA 18-32C X LA12-19C
LA 20	A 20-10 X LA 17-39B
LA 21	LA 19-1A X LA 20-3B

Table 3

Genotype of diploid strains analysed by sucrose gradient centrifugation

Diploid		
LA3	$\frac{a}{\alpha}$	$\frac{ura4\text{-}1}{+}$, $\frac{trp5\text{-}48}{trp5\text{-}48}$, $\frac{lys1\text{-}1}{lys1\text{-}1}$, $\frac{ade2\text{-}1}{ade2\text{-}1}$, $\frac{can1\text{-}100}{can1\text{-}100}$, $\frac{his1\text{-}7}{his1\text{-}315}$, $\frac{sup6\text{-}1\text{-}10}{SUP6\text{-}1}$, $\frac{+}{leu2}$, $\frac{+}{cdc14}$, $\frac{+}{met10\text{-}4}$
LA4	$\frac{a}{\alpha}$	$\frac{ade2\text{-}1}{+}$, $\frac{his}{his}$, $\frac{leu1\text{-}12}{+}$, $\frac{lys1\text{-}1}{lys1\text{-}1}$, $\frac{rad18\text{-}2}{rad18\text{-}2}$, $\frac{+}{ade}$
LA7 LA5	$\frac{a}{\alpha}$	$\frac{ura4\text{-}11}{+}$, $\frac{trp5\text{-}48}{trp5\text{-}48}$, $\frac{lys1\text{-}1}{lys1\text{-}1}$, $\frac{ade2\text{-}1}{ade2\text{-}1}$, $\frac{can1\text{-}100}{can1\text{-}100}$, $\frac{his1\text{-}7}{his1\text{-}315}$, $\frac{sup6\text{-}1\text{-}10}{SUP6\text{-}1}$, $\frac{rad51\text{-}1}{rad51\text{-}1}$, $\frac{+}{leu2}$, $\frac{+}{cdc14}$, $\frac{+}{met10\text{-}4}$

YEPG: 1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol in distilled water.

MC: 0.67% Bacto-yeast nitrogen base without amino acids, 2% D-glucose in 1 litre of distilled water. The following supplements were added from stock solutions to 1 litre of MC: 20 mg of adenine, uracil, arginine, tryptophan, histidine, lysine and methionine; 30 mg of leucine; 350 mg of threonine.

Omission media: MC excluding one of the above supplements.

can: arginine omission medium containing 60 µg/ml canavanine sulphate.

Sporulation media:

F^+: 1% potassium acetate; .1% dextrose, .25% yeast extract, plus supplements as in MC in distilled water.

1% KAc: 1% potassium acetate in distilled water.

2% Bacto-agar was added to the above media for solidified medium.

For growing *E. coli* cells, M9 medium as modified by Miller *et al.* (1976) was used. Modified M9 contains 10% dextrose, 2 g casamino, 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g NaCl, 95 mg $MgCl_2$, 11 mg $CaCl_2$ per litre of water. The dextrose, casamino acids, $MgCl_2/CaCl_2$ and salts were all autoclaved separately.

Dissections and Sporulation

Strains were constructed using standard yeast genetic techniques (Mortimer and Hawthorne, 1969). Diploids were formed by mixing equal amounts of cells of opposite mating types on YEPD. Diploids were cloned by dissecting out a diploid zygote with a micromanipulator. Diploid cells were sporulated by taking an overnight culture from YEPD plates and replica-plating to F^+ sporulation medium. After three days, asci

were digested with 1:10 dilution of glusulase (Endo) and the spores were dissected on YEPD plates. Spore clones were scored by replica-plating to the appropriate medium.

Radioactive Labelling

Yeast RNA and DNA were labelled by growing cells in MC minus uracil medium at 26°C containing 7-15 μCi/ml of [6-^{3}H] uracil (specific activity 21 Ci/mmole) for 2 to 3 generations from an initial concentration of 1 x 10^{7} cells/ml. For radiosensitive strains, the radioactivity was set at 7 μCi/ml to minimize auto-irradiation and selection for radioresistant cells (Hatzfeld,1973). Growth rates for labelled and unlabelled cultures were compared for any indication of auto-irradiation.

T7 phage DNA was labelled and extracted in the following manner. *Escherichia coli* B23 cells were grown at 30°C in 10 mls of modified M9 medium to a concentration of 2 x 10^{7} cells/ml then the DNA was labelled by adding 1 to 3 μCi/ml of [^{14}C] thymidine (spec. act. 50 mCi/mmole). The radioactive thymidine was added in 10 aliquots at 10 minute intervals. When the cells reached 1 x 10^{8} cells/ml they were infected with T7 phage at a multiplicity of 10 (Langman and Paetkau, 1978). After lysis was complete, the solution was made one molar with respect to NaCl, also a 1/2 ml of T7 carrier phage (1 x 10^{12} phage/ml) was added.

The phage were purified by centrifuging at 10,000 rpm for 10 mins. to removed debris and then at 35,000 rpm for 45 mins. in a SW50L rotor to pellet the phage (Studier, 1969). The phage were resuspended in 1.5 mls of T7 Tris salt (.5M NaCl, 0.01 M tris, 1 mM $MgCl_2$ pH 7.5 Miller et al.,1976) and spun again at 10,000 rpm for 10 mins. The phage were further purified by gel filtration through a 18 cm x .8 cm

column of Bio Gel A-50M eluted with T7 tris salt less $MgCl_2$ (Miller *et al.*, 1976).

T7 DNA was extracted either by heating the phage in 5% sarkosyl at 65°C (Kutter and Wiberg, 1968) or by phenol extraction followed by washing with ether (Miller *et al.*, 1976).

Spheroplast Formation

Spheroplasts were formed by the method of Cryer *et al.* (1975), with slight modifications. Yeast cells at a concentration of 3-5 x 10^7 cells/ml were washed once in 0.05 M tetrasodium ethylenediamine-tetraacetate (EDTA) pH 7.5. The washed cells were resuspended in 2% β-mercaptoethanol, 0.02 M EDTA (pH 9.0) and incubated for 10 minutes at room temperature. The cells were spun down and resuspended at the same concentration in 1 M sorbitol, 0.1 M EDTA (pH 7.5) with 2 mg/ml Zymolyase (Kirin Brewery) and incubated at 37°C for 10 minutes. The spheroplasts were spun down at 4,000 x g for 15 minutes and washed once in sorbital-EDTA. Afterwards the washed spheroplasts were gently resuspended in saline-EDTA 0.15 M NaCl, 0.1 M EDTA (pH 8.0) with 100 mg/ml proteinase K (Merck) to destroy nucleases (Gross-Bellard, Oudet and Chambon, 1973).

Isolation of Yeast Lacking Mitochondrial DNA (ρ^o)

Induction of petites with ethidium bromide (EtBr) was done according to the method of Goldring *et al.* (1970). EtBr was added to log phase cells in YEPD medium at a final concentration of 10 μg/ml. The cells were grown overnight and subcultured into EtBr-YEPD once more. Clones that failed to grow on YEPG were tested for the presence of mitochondrial DNA as extra-nuclear fluorescent bodies after staining with 4', 6-diamidino-2-phenylindole (DAPI, Serva) using fluorescence microscopy (Williamson and Fennell, 1975).

Sucrose Gradient Centrifugation

During these studies two types of neutral sucrose gradients were used. The first consisted of a 4.7 ml, 5-20% (w/v) linear sucrose gradient in PLBII buffer (0.1 M NaCl, 0.02 M EDTA pH 7.0) (Blamire *et al.*, 1972). The second was a 4.8 ml 15-30% sucrose gradient in 1% sarkosyl, 0.01 M EDTA, 0.1 M tris-HCl and 1 M NaCl at pH 8.0, (Petes and Fangman, 1972).

Alkaline sucrose gradients were made up of 15-30% linear sucrose gradient in 1% sarkosyl, 0.9 M NaCl, 0.3 M NaOH, 0.01 M EDTA at pH 12.5 (Petes and Fangman 1972). A 0.1 ml, 1% sucrose in 1 M NaCl, 0.01 M EDTA, solution was overlaid onto the gradient and acted as a buffer zone between the proteinase K and the high pH of the gradient.

For all gradients, a 0.1 ml suspension of spheroplasts was layered onto a lysis layer consisting of either 0.1 ml 1% sarkosyl or 50 µl of 5% sarkosyl in 0.1 M EDTA, 0.01 Tris-HCl, 1 M NaCl (pH 8.0). Lysis was carried out for 15 minutes at room temperature, after which 0.1 ml of ^{14}C-T7 DNA was placed on top of the gradient. Rotors used and centrifigation speeds and times are given in the legends. The temperature during the runs was approximately 5°C.

The gradients were fractionated (0.2 ml) using an ISCO gradient fraction collector. RNA was hydrolysed with 1 ml of 0.3 M KOH at 37°C for 12 hrs. Samples were made ice cold, neutralized with 0.6 M HCl and 0.5 ml of bovine serum albumin (200mg/ml) was added as carrier (Jacobson *et al.*, 1975). DNA and albumin were precipitated by making the solution 10% with respect to trichloracetic acid (TCA) and collected on glass-fiber filters (Whatman GF/C). The filters were washed four times with cold 5% TCA and twice with cold 95% ethanol.

Dried filters were counted in a liquid scintillation counter using Aquasol® (New England Nuclear) as the scintillant. Toulene based scintillation fluid with 0.5% PPO and 0.01% POPOP was also used and gave similar results. All samples were counted for 5 minutes which gave less than 10% counting error. Recovery of radioactivity on the gradient ranged from 75% to 95%.

The molecular weight of each fraction was calculated by its position relative to T7 DNA using the equation $(d_1/d_2) = (M_1/M_2)^x$, (Burgi and Hershey, 1963), where d_1 and d_2 are the distances migrated by yeast and T7 DNA respectively in a sucrose gradient and M_1 and M_2 are the molecular weights of yeast and T7 DNA in those fractions. The value for the coefficient x was set at 0.41 for neutral gradients (Resnick and Martin, 1976).

Number average molecular weight (Mn) was calculated using the equation $Mn = \sum_{y}^{z} ri / \sum_{y}^{z} ri/Mi$ where ri is the amount of radioactivity in the i^{th} fraction and Mi the molecular weight of that fraction (Lett *et al.*, 1970). Number average molecular weight was calculated from the low point on the small molecular weight side of the profile to the plateau on the high molecular weight region of the profile. Inaccuracies in calculating Mn can occur when determining the position on the low molecular weight side of the DNA profile. As can be seen in the above formula, any inaccuracies in determining ri or Mi in the first few fractions after the starting zone can greatly influence the final Mn value (Lett *et al.*, 1970). Also, any low molecular weight material not of chromosomal origin overlapping in this region would influence calculations of Mn.

DNA Purification, Denaturation and Alkali Treatment

Spheroplasts in saline-EDTA with proteinase K were lysed by the

addition of 25% sarkosyl to a final concentration of 1%. The cells were incubated for 3 hrs at 37°C until lysis was complete, then the lysate was heated at 60°C for 1/2 hr. (Cryer *et al.*, 1975)

One method of purification was the use of CsCl isopycnic centrifugation using the method of Skalk and Bartl (1974), which allows one to isolate high molecular weight DNA if precautions are taken against shearing. To 4 ml of lysate (approx. 4×10^8 cells) 5.2 gms of CsCl was added giving a refractive index of 1.4000. The CsCl was dissolved by gentle shaking (<25 rpm) in a 200 ml flask. The solution was transferred with a large bore pipet to the centrifuge tubes and spun at 33,000 rpm for 48 hours in SW50L rotor at 20°C. The gradient was fractionated by displacing the CsCl with ethylene bromide or a saturated solution of CsCl. A 10 µl sample of each fraction was analysed for alkali-stable acid precipitable radioactivity. The fractions containing radioactivity were pooled, diluted with SSC (0.15 M NaCl 0.015 M Na citrate pH 7.0) and dialysed against SSC at 4°C.

DNA was also purified adapting the technique of Reynolds (1978). Spheroplasts were resuspended in 2 x SSC, 0.002 M EDTA (pH 8.0) and 100 µg/ml proteinase K at a concentration of approximately 4×10^7 cells/ml. Then an equal volume of 2% sarkosyl was added and lysis was continued for 3 hrs at 37°C. Two volumes of SSC saturated phenol were added to the lysate and the proteins extracted by gentle rolling for 2 hrs at room temperature to mix the phases. After removal of the phenol phase, the aqueous phase was extracted twice with water saturated ether for 20 minutes each time. The ether layer was removed and the residual ether was removed by incubating the solution at 35°C. The aqueous phase was dialysed against SSC containing 15 mM $MgCl_2$ (pH 7.6) at 4°C overnight.

DNA was denatured at neutral pH by dialysing against 95% formamide, 5% 0.015 M EDTA (pH 7.0) for 3 hrs at 4°C with two changes. The DNA formamide solution was heated to 37°C for 2 hrs, then dialysed against SSC-6% formaldehyde (pH 7.0) at 4°C, (with 3 changes) to prevent renaturation and subsequently against SSC (Verly and Paquette, 1972).

To break alkali-labile bonds in DNA the following treatment was performed. DNA in SSC buffer was made 0.3 M with NaOH and incubated at 25°C for 8 to 16 hours. Untreated DNA samples had an equivalent volume of SSC added and both treated and untreated samples were denatured as before with formamide. The NaOH treated DNA was exposed to formamide, as the control sample, to ensure that both samples received the same amount of handling.

Assay for Apurinic Sites in DNA

The assay measures nicking in DNA after treatment with an endonuclease which specifically cuts near an apurinic or apyrimidinic site. Endonuclease VI from *E. coli*, an AP endonuclease (Gossard and Verly, 1978) was kindly provided by Dr. A. R. Morgan. This batch of enzyme also has exonuclease activity, but under the conditions used the exonuclease activity was very low (B. Futcher, pers. comm.).

16 µg of endonuclease VI was added to 1 ml of phenol purified yeast DNA (1 µg/ml in SSC; 15 mM $MgCl_2$ at pH 7.6 with 100 µg/ml bovine serum albumin). The mixture was incubated at 37°C for 15 minutes and the reaction was stopped by chilling and the addition of 0.1 volume of 2% sodium dodecyl sulfate (SDS), 100 mM EDTA and 1000 µg/ml proteinase K. The DNA solution was deproteinized with phenol and denatured by the formamide and formaldehyde method. The denatured DNA in SSC was spun on 4.7 ml 5-20% neutral sucrose gradients to determine the extent of strand breakage.

Ultraviolet (U.V.) Irradiation

For U.V. survival curves appropriate dilutions of cells from an overnight growing culture were plated on YEPD plates and irradiated in the dark. The U.V. lamp gave a dose rate of 1.4 Joules/m^2/sec as determined by a Latarjet Dosimeter. Plates were wrapped in aluminum foil and incubated for 5 days at 26°C before scoring.

Gamma Ray Irradiation

Cells to be irradiated for sedimentation velocity experiments were suspended in distilled H_2O and tubes were placed in beakers with ice water. The source of x-rays was ^{60}Co γ-cell (Atomic Energy of Canada, Ltd.), with a dose rate of approximately 2 krads/min. Spot tests for survival were done by placing a drop of yeast cells (approx. 10^6 cells) on a YEPD plate or replica-plating spore clones and irradiating for 20 minutes. Survival curves were done by plating an appropriate dilution of an overnight culture on YEPD plates and irradiating the plates in the γ-cell.

Methyl methanesulfonate (MMS) Survival Curves

Cells were grown overnight to approximately 1 x 10^7 cells/ml in YEPD. The culture was washed twice with 0.05 M potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. Redistilled MMS(Terochem) was added to a final concentration of 0.4%. After various times in contact with MMS, a sample of cells was removed, spun down and resuspended in cold 10% $Na2S_2O_3$ for 10 mins to inactivate the MMS. Appropiate dilutions were made and plated on YEPD plates to measure survival.

RESULTS

Survival Curves

Typical γ-ray survival curves for the diploid strains used in this study are found in figure 1. As expected both *rad51-1* and *rad18-2* strains are very sensitive to the killing effects of γ-rays. At higher doses *rad18* diploids show a resistant tail not seen in *rad51* strains. It should also be noted there is no significant difference between a respiratory deficient (ρ^-) strain and a ρ^+ strain of *rad51*. It is reported that *rad51* strains are slightly sensitive to ultraviolet light (Nakai and Matsumato, 1967) whereas *rad18* strains are very sensitive to U.V. (Resnick, 1969). Both *rad51* and *rad18* strains are methyl methanesulfonate sensitive (Brendel *et al.*, 1970). These results have been confirmed with the exception of MMS sensitivity of *rad18-2* strains (Mowat, unpublished data; Quah, pers. comm.).

Sucrose Gradients of DNA from Wild Type Cells

Figure 2 shows sucrose gradient profiles of DNA from non-irradiated wild type yeast cells. One profile is from a log phase diploid the other two profiles are from haploids, one taken from a log phase culture the other from a stationary culture. The gradients show a conventional yeast DNA sedimentation profile (Blamire *et al.*, 1972; Resnick and Martin, 1976). The dashed line represents the profile of T7 DNA. T7 phage DNA has a molecular weight of 2.5×10^7 daltons (Freifelder, 1970). Resnick and Martin (1976) have shown that 5-20% neutral sucrose gradients are isokinetic. Since the conditions used with these gradients were similar to theirs, it is valid to calculate the number average molecular weight (Mn) of yeast DNA based on its relative position to T7 DNA. The Mn for

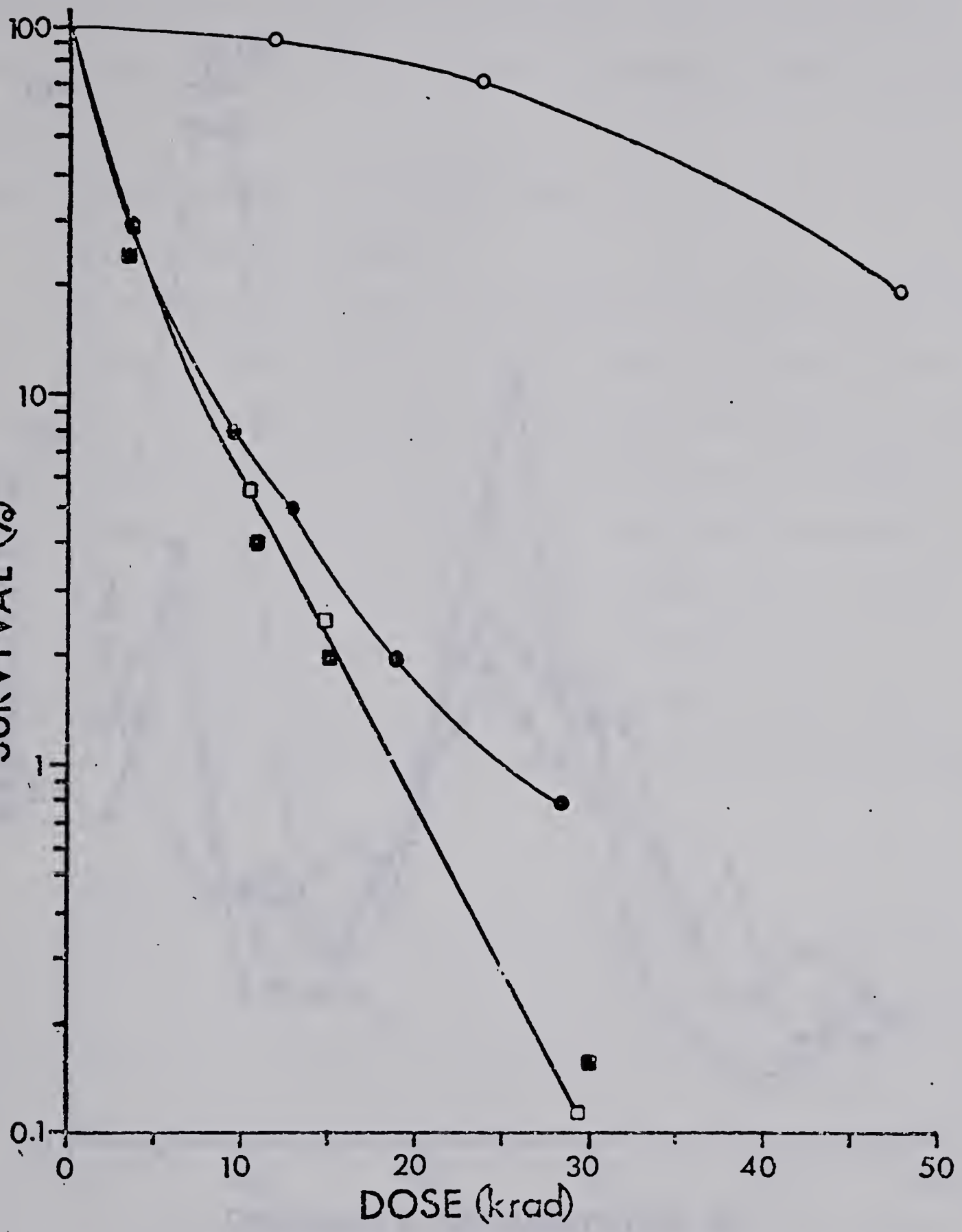


Fig. 1. γ-ray survival of diploid yeast,

(○) LA 3 *RAD⁺/RAD⁺*; (●) LA 4 *rad18-2/rad18-2*

(□) LA 5 *rad51-1/rad51-1* ρ⁻; (■) LA 5 ρ⁺.

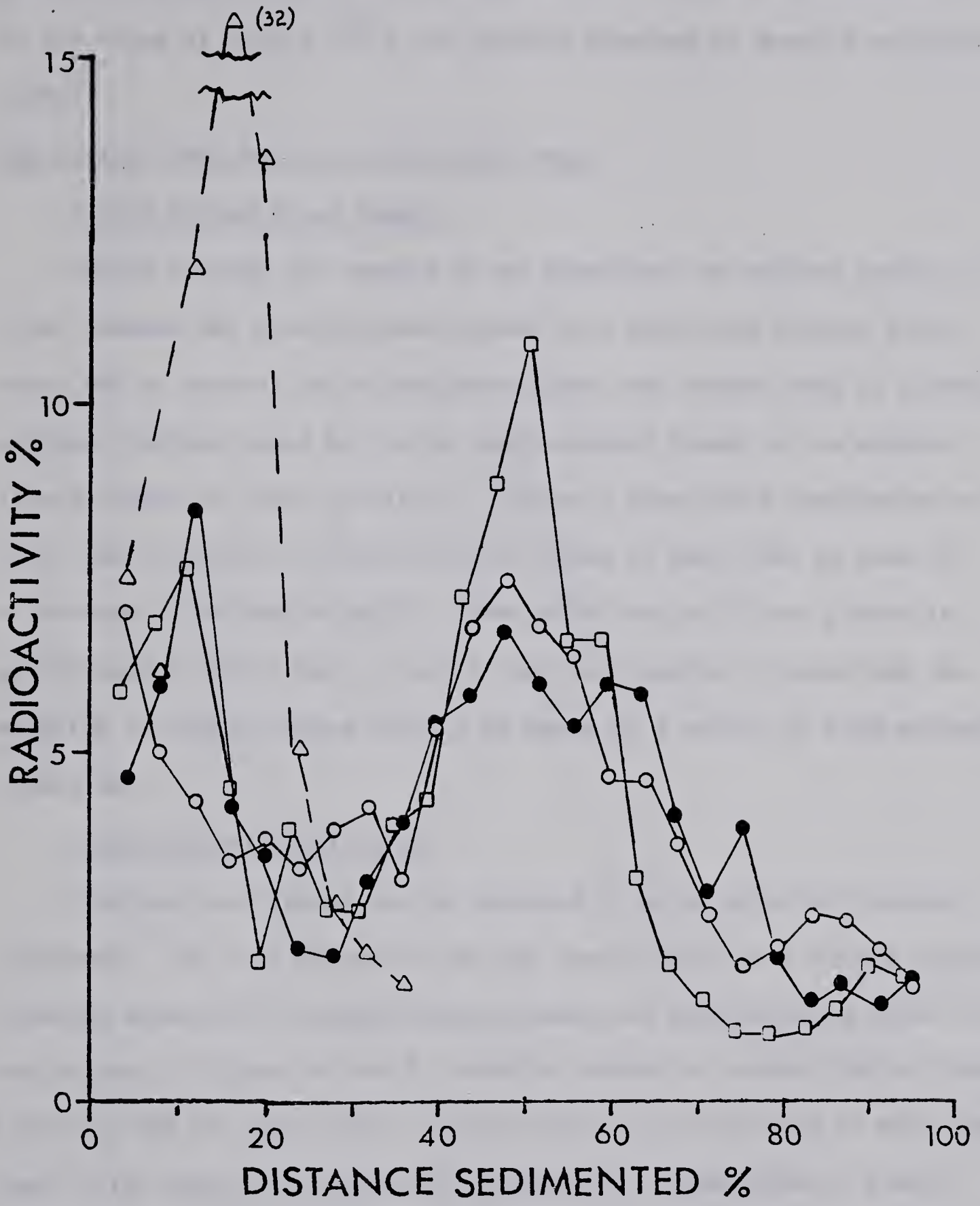


Fig. 2. Neutral sedimentation pattern of DNA from three wild type yeast strains. The cells were labelled as described in the Materials and Methods section except that the haploid strain KF179-17A ρ^- was labelled with 4 µCi/ml of ^{3}H-uracil. Spheroplasts were formed as described in the Methods section and layered onto 5-20% neutral sucrose gradients. Centrifugation was at 10,000 rpm for 14 hrs in a SW50L rotor. (□) diploid strain LA3 ρ^- from a log phase culture. (○) haploid strain KF179-17A ρ^- from a log phase culture. (●) haploid strain KF179-17A ρ^- grown to stationary phase. (△) T7 phage DNA. Total counts on each gradient were (□) 11,963 cpm; (○) 559 cpm; (●) 1086 cpm; (△) ^{14}C-2791 cpm.

the diploid strain shown is 2.5×10^8 daltons. This is comparable to the value of $3.03 \times 10^8 \pm .39$ daltons obtained by Resnick and Martin (1976).

DNA Strand Break Repair in Wild Type Yeast

Double-Strand Break Repair

Figure 3 shows the results of an experiment to measure repair of γ-ray induced DNA double-strand breaks in a wild type diploid yeast. Since DNA at neutral pH is double-stranded, any breaks seen on a neutral sucrose gradient would be due to double-strand breaks or to single-strand breaks in close proximity. Figure 3 shows that irradiation of yeast cells results in double-strand breaks in their DNA as seen by a decrease in molecular weight. When wild type cells are placed in growth medium (YEPD) for 3.0 hours they are capable of repairing the majority of double-strand breaks, as shown by a return to high molecular weight DNA.

Single-Strand Break Repair

Single-strand breaks can be detected by using alkaline sucrose gradients. The high pH results in DNA denaturation into single strands allowing detection of single-strand breaks and alkali-labile bonds. As can be seen in figure 4a and b, ionizing radiation causes single-strand breaks in DNA of yeast cells. Post-irradiation incubation of wild type yeast cells results in repair of single-strand breaks and/or alkali-labile bonds. This is seen by an increase in size of single stranded DNA from the size immediately after irradiation towards the size of DNA from unirradiated cells.

DNA Strand Break Repair in *rad51-1* Strains

Double-Strand Break Repair

Since *rad51* is in the same epistasis group as *rad52* (Game and Mortimer,

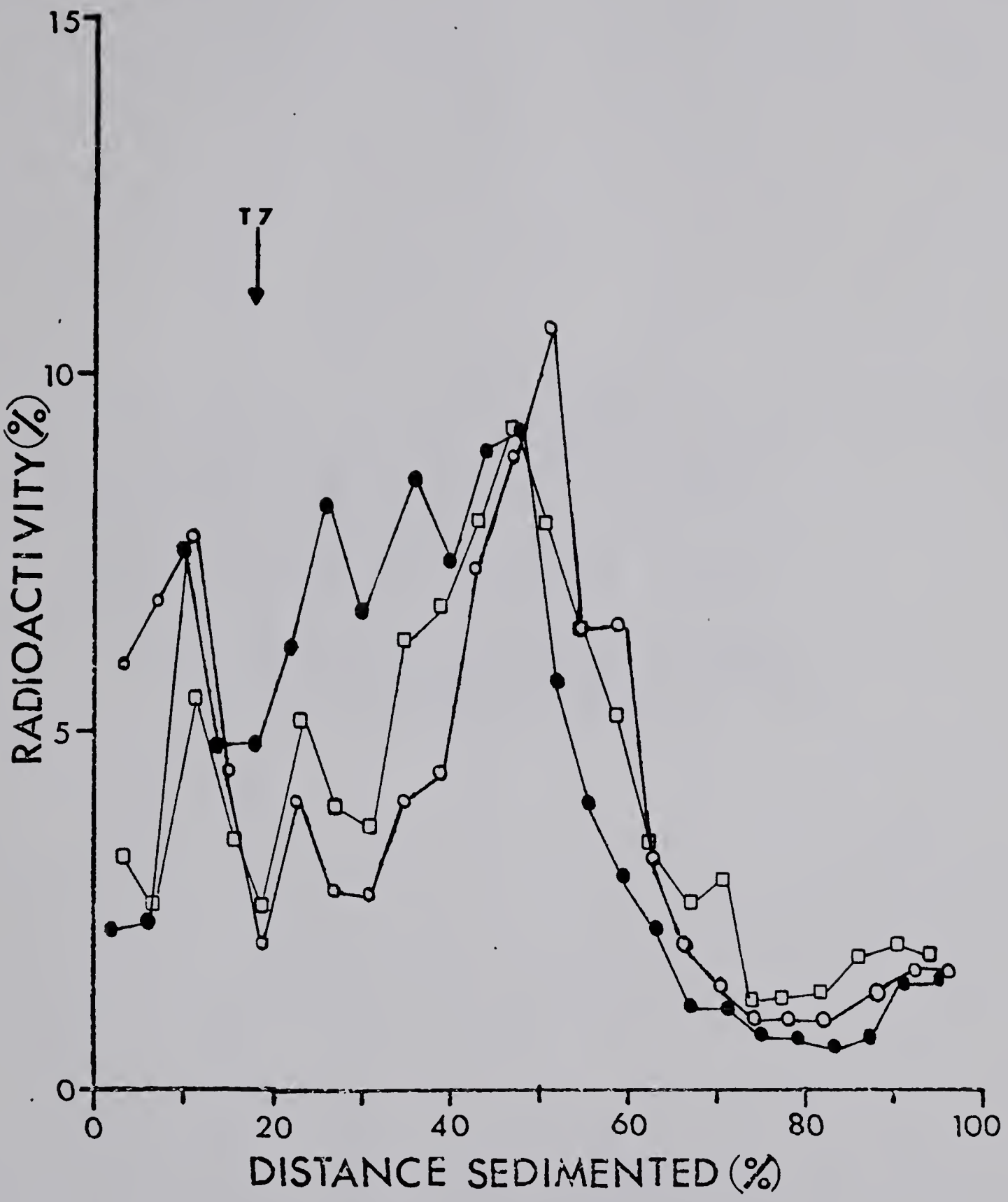
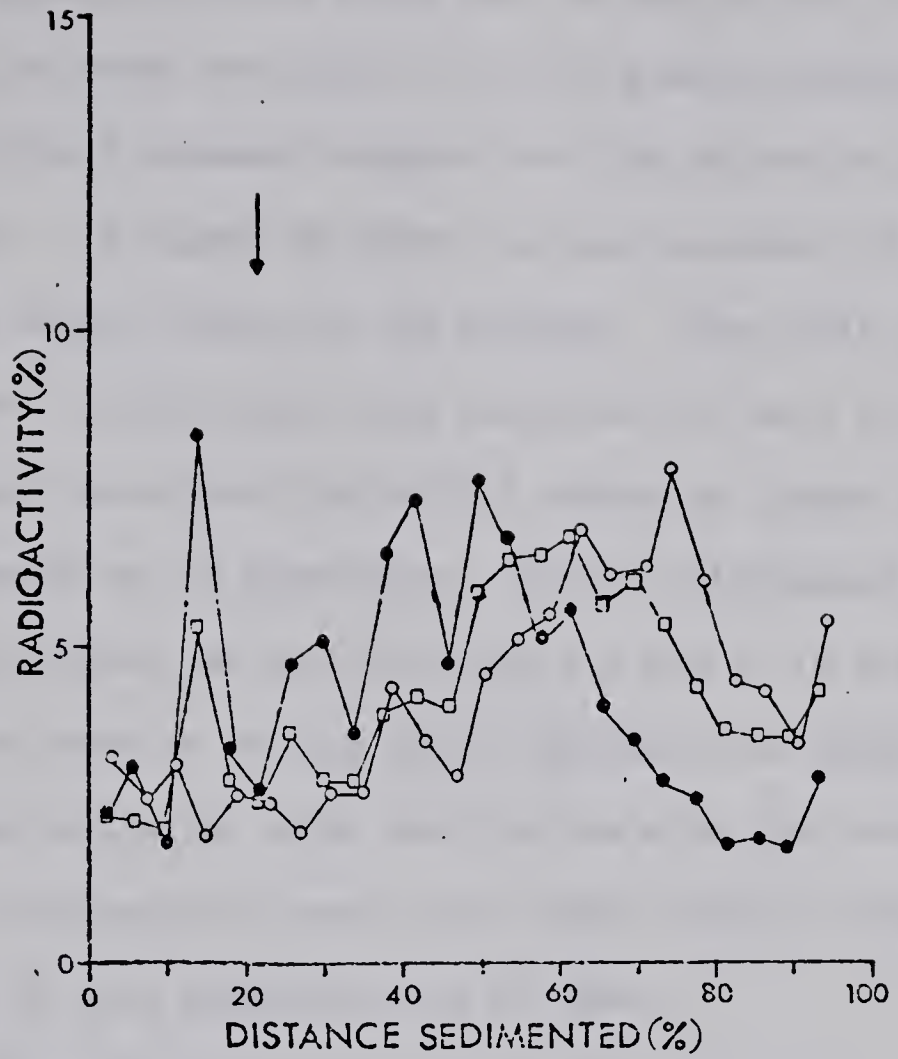


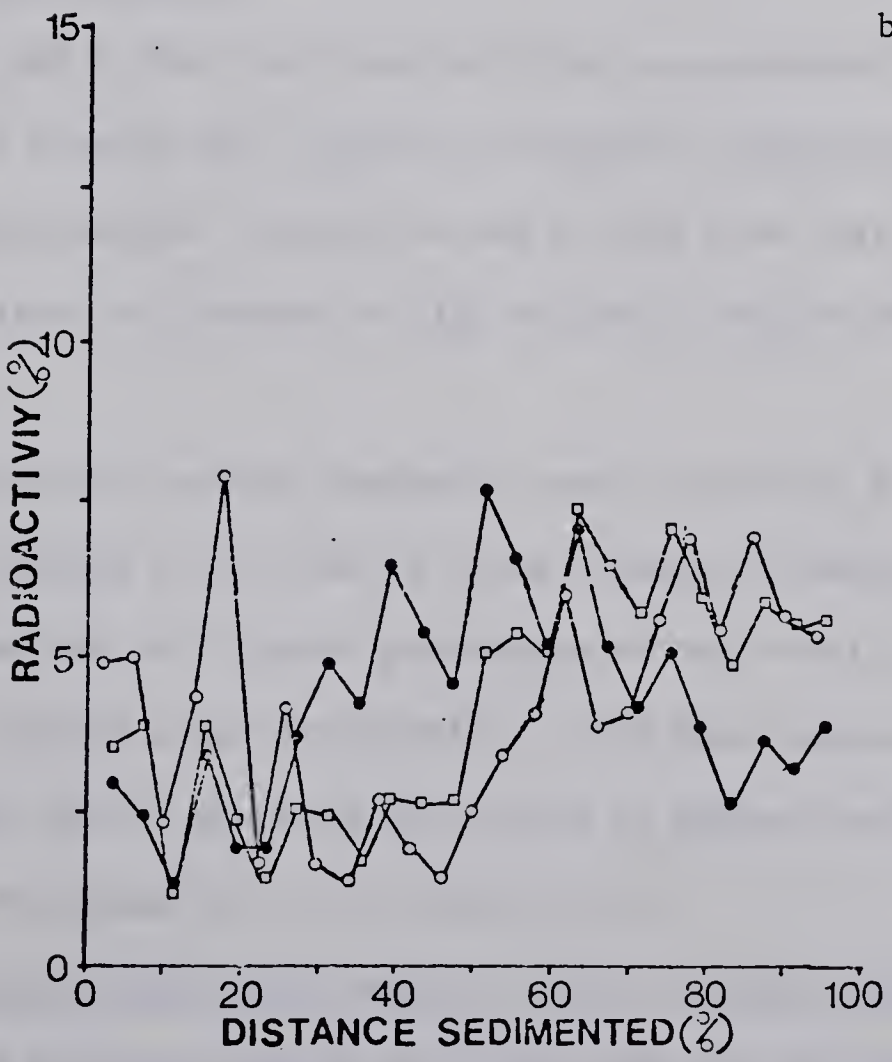
Fig. 3. Neutral sedimentation pattern of chromosomal DNA from the wild type diploid strain LA3 (ρ^-,*RAD*$^+$/*RAD*$^+$) after 30 krads. Cells receiving post-irradiation incubation in YEPD were irradiated three hours prior to cells not to be incubated after irradiation. Non-incubated cells were kept on ice during this period. Cells were made into spheroplasts and layered onto a 5-20% neutral sucrose gradient. Centrifugation was at 10,000 rpm for 14 hrs in a SW50L rotor. (O) no irradiation; (●) 30 krads only; (□) 30 krads + 3 hrs post-irradiation in YEPD. The total ^{3}H cpm on each gradient is (O) 11,963 cmp; (●) 11,664 cpm and (□) 12,668 cpm.

Fig. 4a and b. Alkaline sedimentation pattern of DNA from diploid strain LA3 (RAD^{+}/RAD^{+}, ρ^{-}) after 20 krads. Irradiation and post-irradiation incubation were as given in figure 3 except dose. Spheroplasts were layered onto a 15-30% alkaline sucrose gradient. Centrifugation was at (a) 11,000 rpm or (b) 12,000 rpm for 26 hrs in a SW50.1 rotor. The total ^{3}H cpm on each gradient was (a) (o) 3904 cpm; (●) 5900 cpm; (□) 4944 cpm; (b) (o) 3115 cpm; (●) 5471 cpm and (□) 4575 cpm.

a


15
10
5
0
RADIOACTIVITY(%)
0
20
40
60
80
100
DISTANCE SEDIMENTED(%)

b


15
10
5
0
RADIOACTIVIY(%)
0
20
40
60
80
100
DISTANCE SEDIMENTED(%)

1974), mutants carrying *rad51* are expected not to show double-strand break repair. This expectation was borne out, as can be seen in figures 5a and b. Figure 5a shows that with 3.5 hours post-irradiation incubation there is very little movement towards the high molecular weight region of the gradient. In figure 5b there was some movement of DNA to the high molecular weight region of the gradient. The shift is to a lesser extent than seen in wild type cells incubated for only 3 hrs (figure 3). The slight repair seen with the *rad51-1* strain in figure 5b may be due to some revertants in the population. Another difference between wild type and *rad51* strains, as seen in figures 3 and 5, is the large amount of radioactivity seen at the top of the gradients of *rad51* strains. The amount is almost twice the level seen on the wild type curves. This low molecular component was seen in all *rad51* strains looked at. The possible cause of this component will be seen.

Single-Strand Break Repair

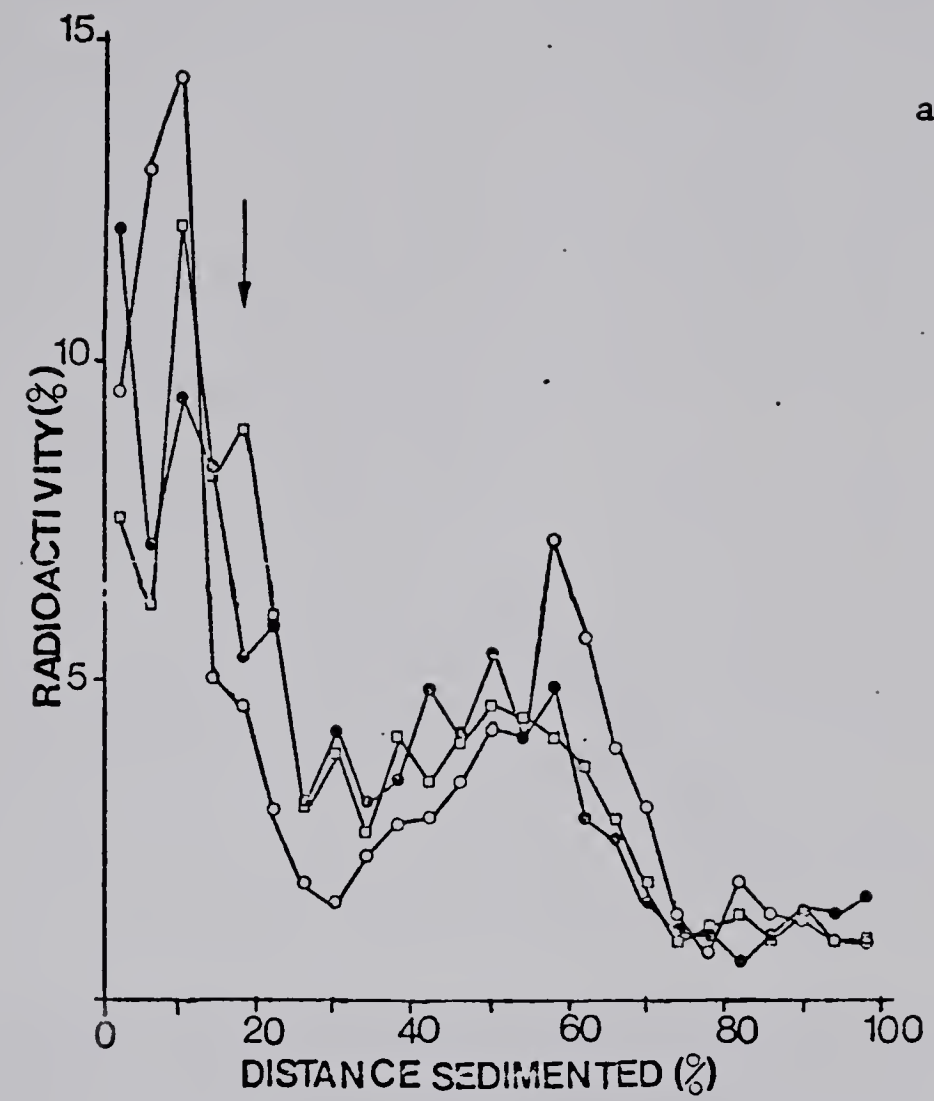
Figure 6a and b show the results of two experiments to determine whether *rad51-1* strains are capable of repairing single-strand breaks induced by γ-irradiation. Figure 6a and b both show that post-irradiation incubation results in a return to high molecular weight DNA in *rad51-1* strains.

The low molecular weight component seen on neutral gradients of *rad51* strains (figure 5) is seen on these alkaline gradients for the zero dose curves but at a larger percentage of the total counts. This component was reduced after irradiation. This peak causes the high molecular weight DNA on the zero dose curve to appear lower when expressed as percentage of total radioactivity.

Apurinic and Apyrimidinic Site Repair in Wild Type and *rad51* Strains

If the low molecular weight component seen in gradients of *rad51*

Fig. 5a and b. Neutral sedimentation pattern of DNA from diploid strains (a) LA7 and (b) LA5 (*rad51-1/rad51-1*, ρ^-) after 30 krads. Irradiation and post-irradiation procedures are as given in figure 3. Spheroplasts were layered onto a 5-20% neutral sucrose gradient. Centrifugation was at 10,000 rpm for 14 hrs in a SW50L rotor. (o) 0 krads; (●) 30 krads only; (□) 30 krads + 3.5 hrs post-irradiation incubation in YEPD. The total ^3H cpm on each gradient was (a) (o) 3550 cpm; (●) 2881 cpm; (□) 3374 cpm; (b) (o) 2839 cpm; (●) 1959 cpm and (□) 1643 cpm.


a
15
10
5
0
RADIOACTIVITY(%)
0
20
40
60
80
100
DISTANCE SEDIMENTED (%)

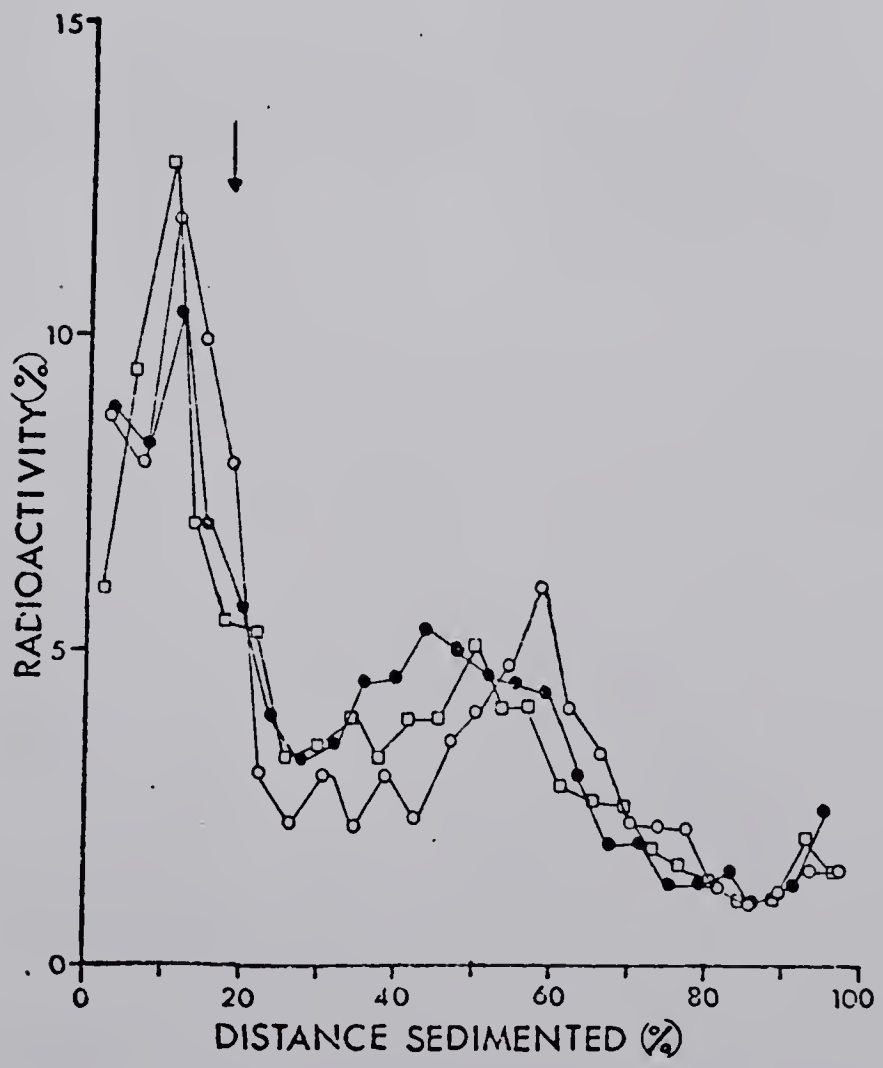
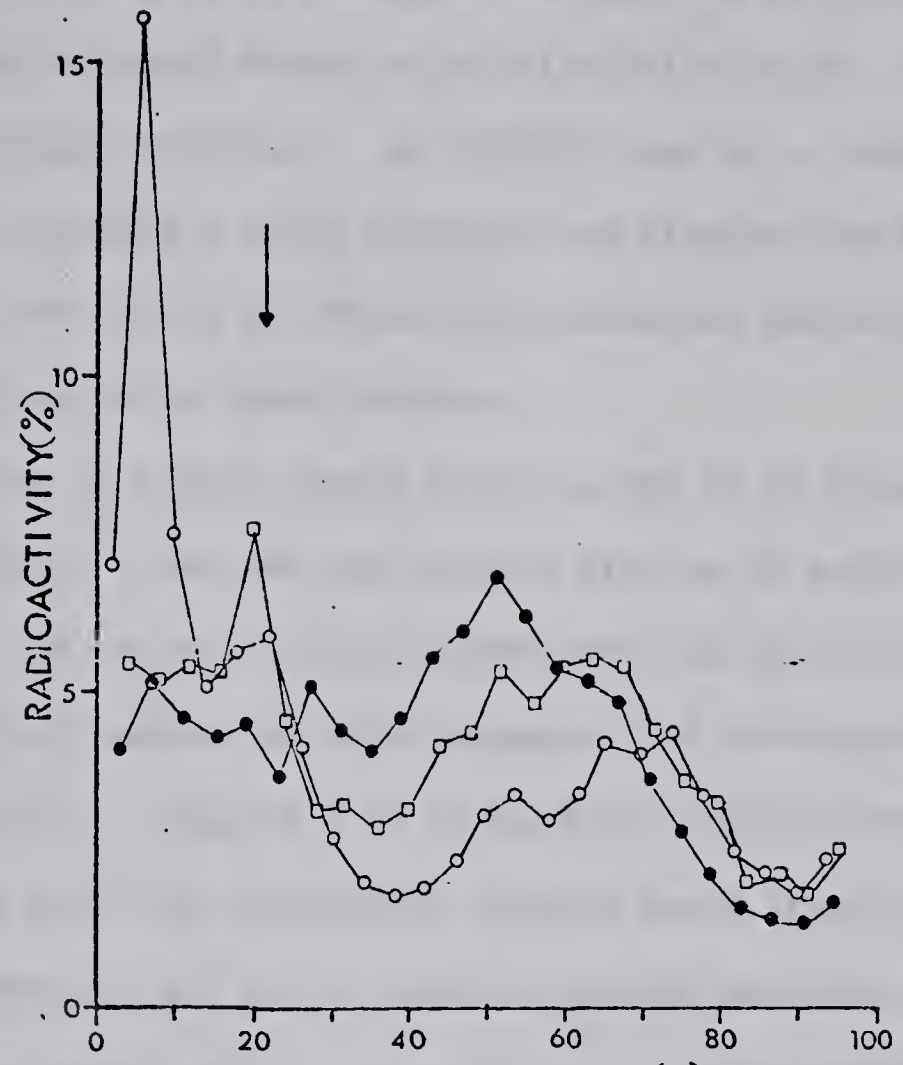

b
15
10
5
0
RADIOACTIVITY(%)
0
20
40
60
80
100
DISTANCE SEDIMENTED (%)

Fig. 6a and b. Alkaline sedimentation pattern of DNA from diploid strain LA7 (*rad51-1/rad51-1*, ρ^-) after 20 krads. Irradiation and post-irradiation are as given in figure 4. Spheroplasts were layered onto a 15-30% alkaline sucrose gradient. Centrifugation was at (a) 11,000 rpm or (b) 12,000 rpm for 26 hrs in a SW50.1 rotor. The total ^3H cpm on each gradient was (a) (o) 7072 cpm; (●) 6851 cpm; (□) 6938 cpm; (b) (o) 4830 cpm; (●) 3999 cpm and (□) 3937 cpm.

a


RADIOACTIVITY(%)
15
10
5
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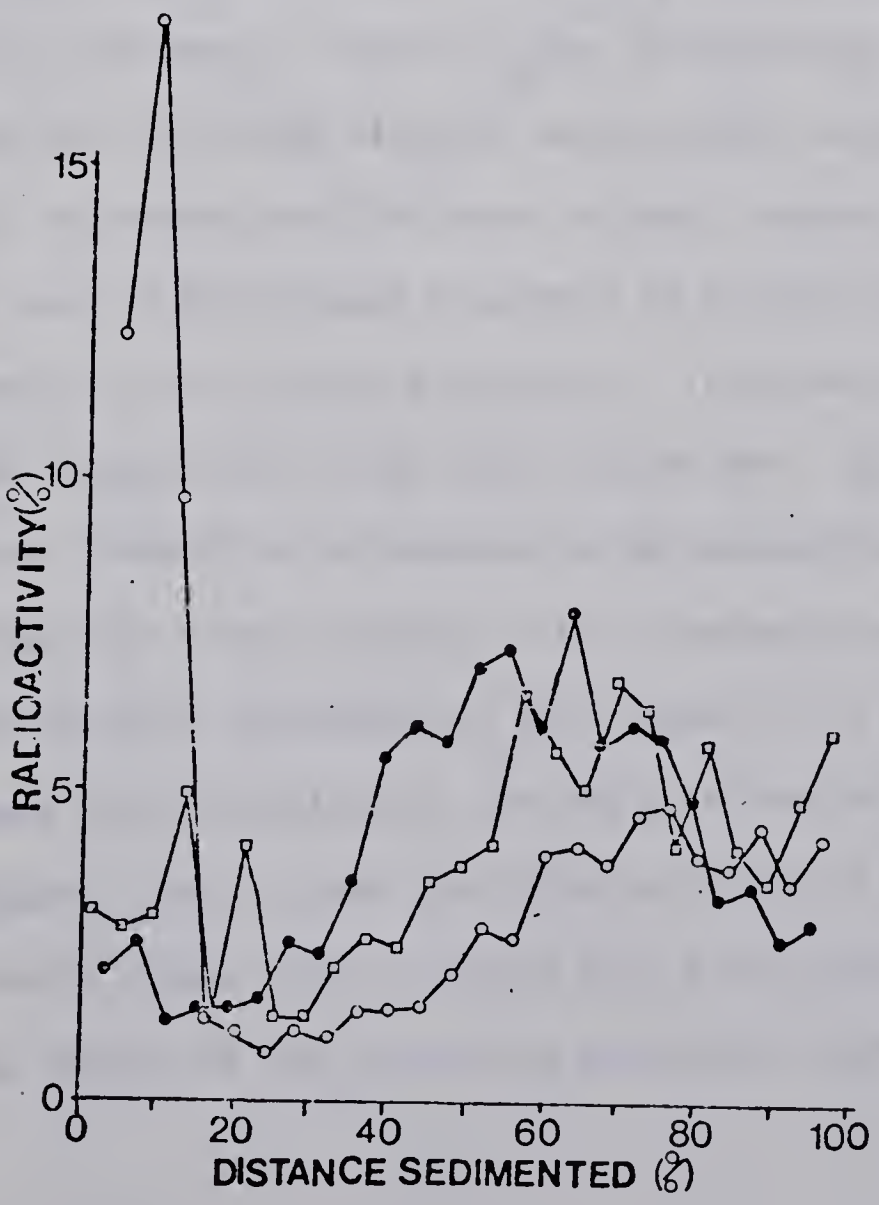
20
40
60
80
100
DISTANCE SEDIMENTED(%)

b


RADIOACTIVITY(%)
15
10
5
0
0
20
40
60
80
100
DISTANCE SEDIMENTED (%)

strains is DNA, then it is possible that the DNA in *rad51* strains contain intrinsic single-strand breaks or alkali-labile bonds. This may reflect a lack of a repair component. Ho (1975b) came to a similar conclusion that strains carrying a *rad52* mutation had single-strand breaks in their DNA when she was unable to obtain high molecular weight DNA on alkaline sucrose gradients with these strains.

One source of alkali labile bonds in DNA is AP sites (Lindahl and Andersson, 1972). Yeast DNA was treated with an AP endonuclease to nick at AP sites. To prevent alkaline hydrolysis in the control the DNA was denatured at neutral pH with formamide and prevented from reannealing with formaldehyde. Figures 7 to 12 show the results from phenol isolated DNA from wild type and *rad51/rad51* diploid yeast treated with AP endonuclease, denatured and run on neutral sucrose gradients. DNA from non-irradiated yeast cells show no AP endonuclease sensitive sites (figures 7 and 10). The increase in counts in the low molecular weight region of the curve (in fig. 7, 0-10%) after AP endonuclease treatment may reflect the action of the exonuclease activity on small pieces of DNA. The variation in size of DNA between gradients is probably due to non-specific shearing during phenol extraction. Irradiation of yeast cells results in the introduction of AP sites in the DNA. This can be seen in figures 8 and 11 as a decrease in the proportion of higher molecular weight DNA after treatment with AP endonuclease. Four hours post-irradiation incubation in YEPD results in a repair of AP endonuclease sensitive sites in the DNA from both wild type and *rad51* strains. Figures 9 and 12 show that treatment with AP endonuclease results in little change from untreated DNA in the high molecular weight regions. The results of the preceeding experiments show that AP sites

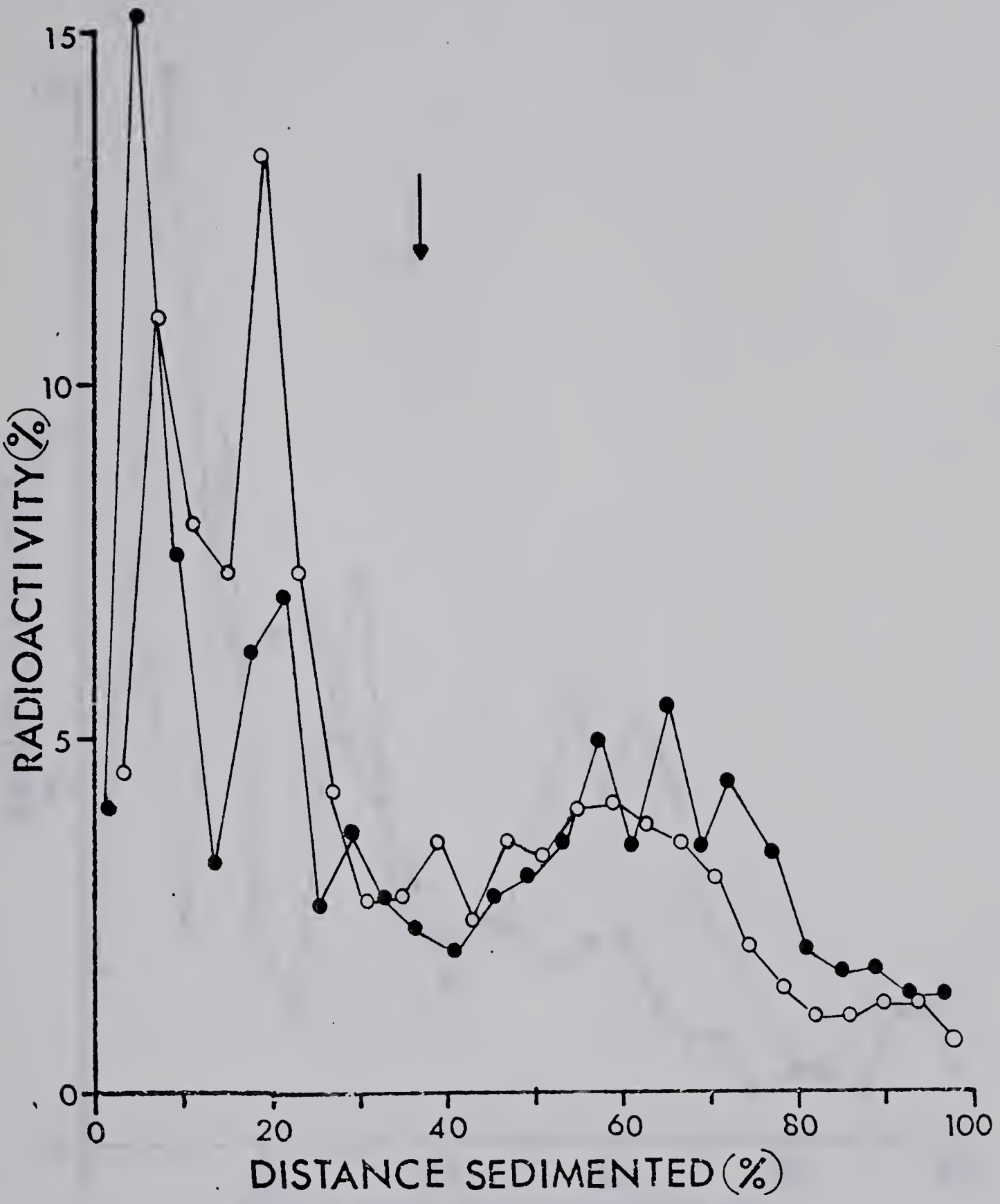


Fig. 7. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from diploid strain LA7 (*rad51/rad51*, ρ^-) treated with AP endonuclease. DNA was isolated by the phenol method and treated or not treated with AP endonuclease as described in the Materials and Methods section. The DNA was further purified with phenol and denatured with formamide and formaldehyde as described in Methods section. T7 DNA was denatured in the same manner. The DNA was applied to a 5-20% neutral sucrose gradient and spun at 35,000 rpm for 2 hrs in a SW50L rotor. (O) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. The total ^3H cpm on each gradient was (O) 3265 cpm; (●) 2083 cpm.

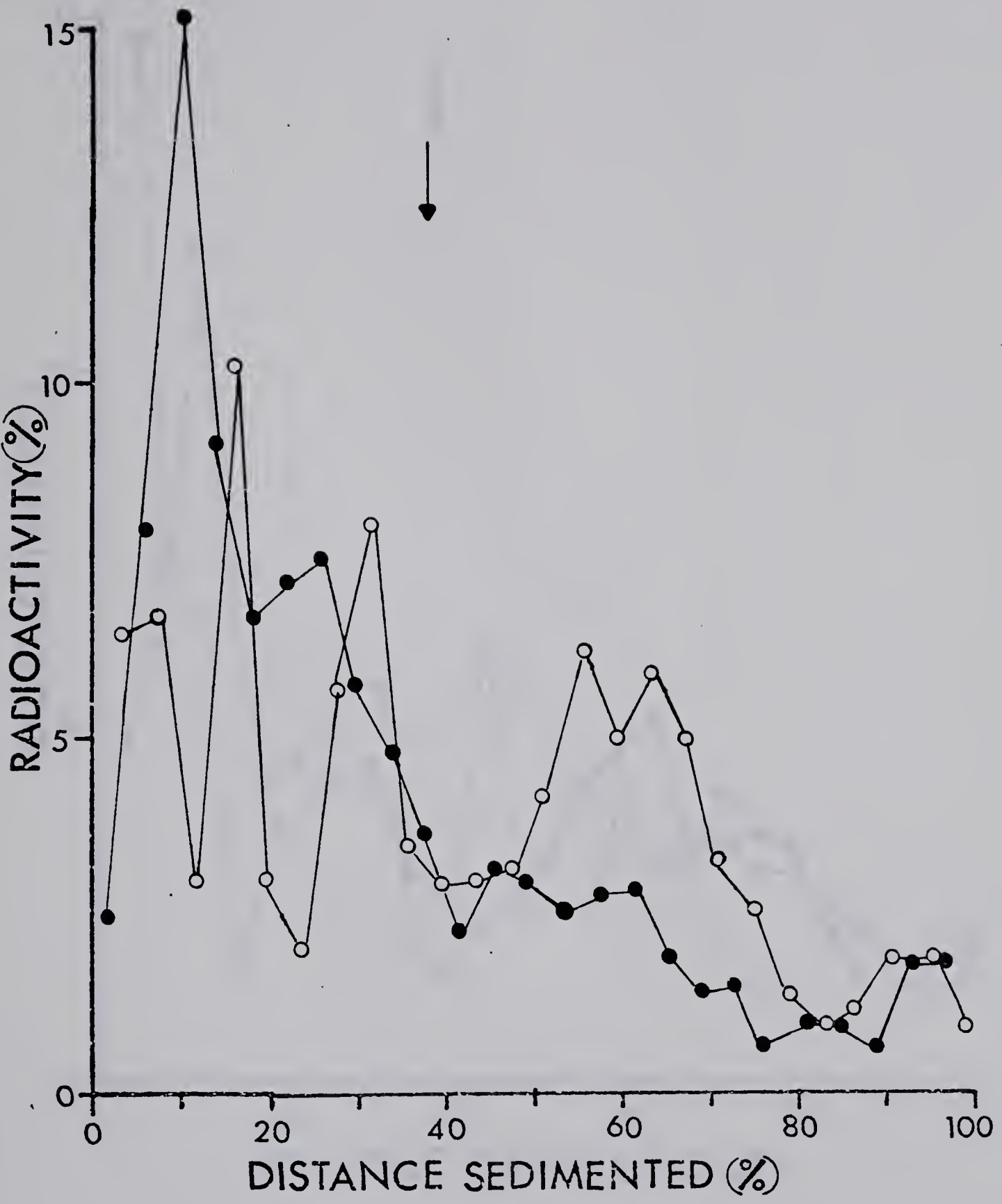


Fig. 8. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from diploid strain LA7 (*rad51/rad51*, ρ^-) after 30 krads treated with AP endonuclease. The procedure was as described in figure 7. (○) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. The total ^3H cpm on each gradient was (○) 2369 cpm; (●) 1816 cpm.

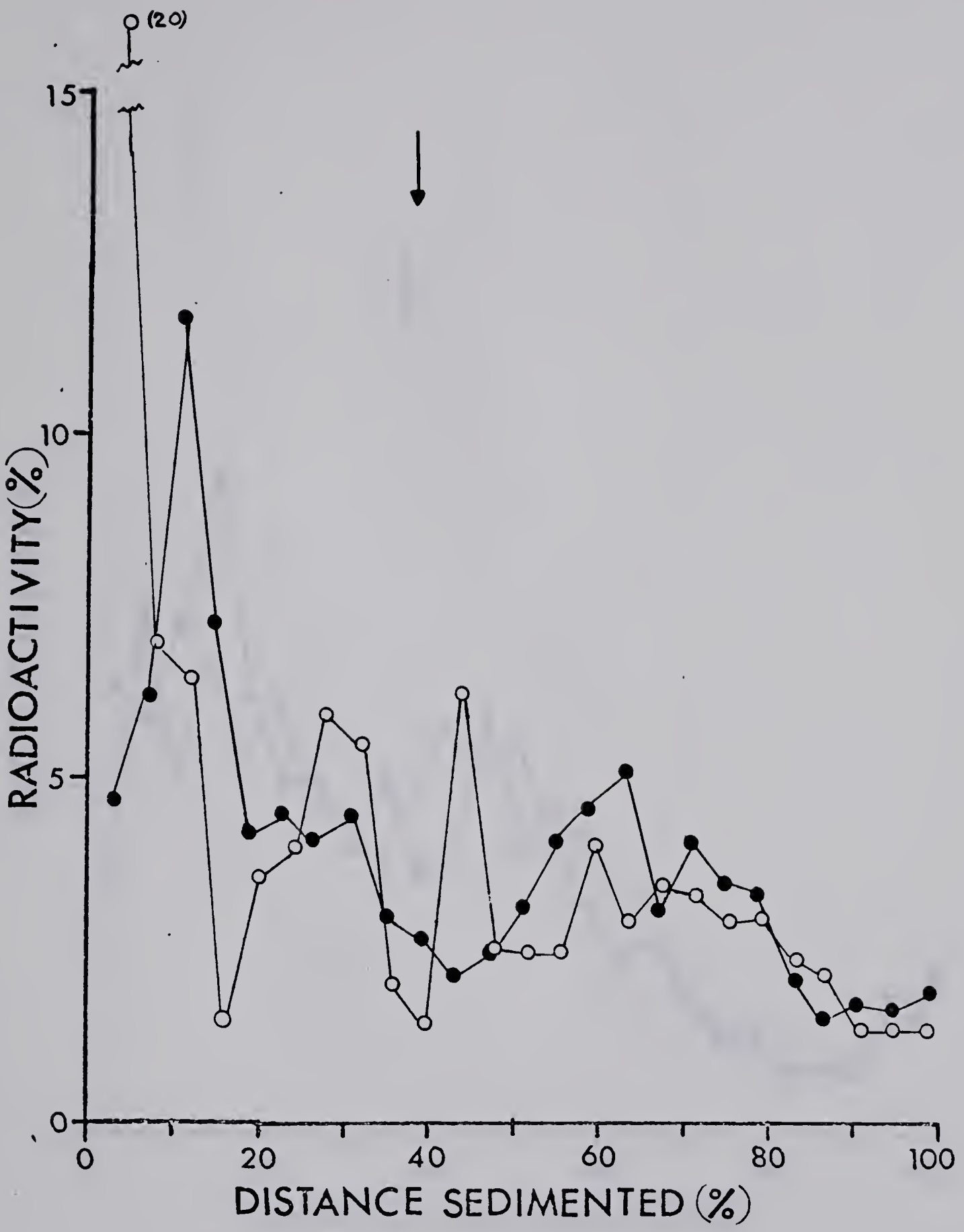


Fig. 9. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from diploid strain LA7 (*rad51/rad51*, ρ^-) after 30 krads + 4 hrs post-irradiation incubation in YEPD, treated with AP endonuclease. The procedure was as given in figure 7. (o) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. Total ^3H cpm on each gradient was (o) 1274 cpm and (●) 1423 cpm.

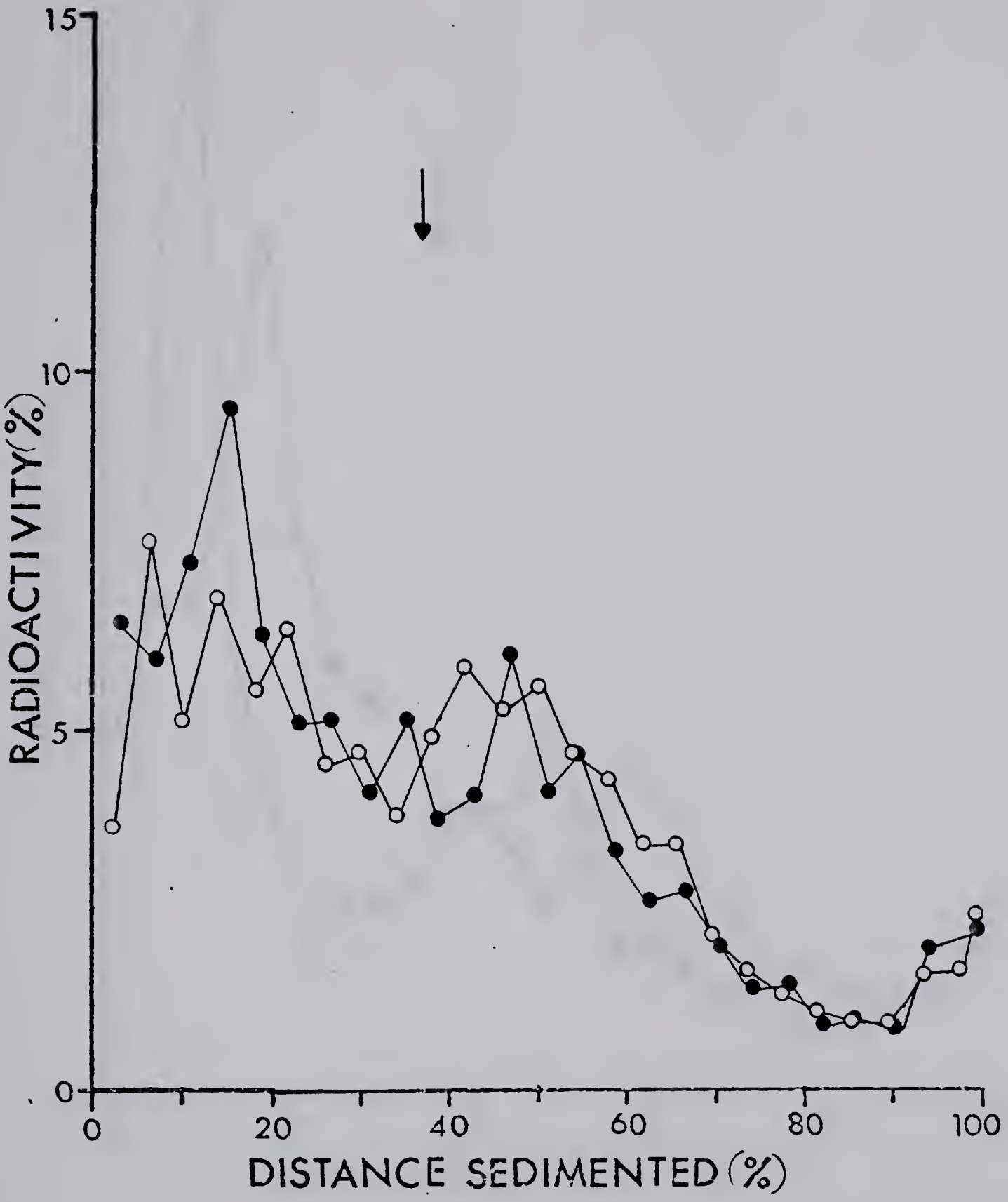


Fig. 10. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from diploid strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$, ρ^{-}) treated with AP endonuclease. The procedure was as described in figure 7. (○) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. The total ^{3}H cpm on each gradient was (○) 1855 cpm; (●) 1751 cpm.

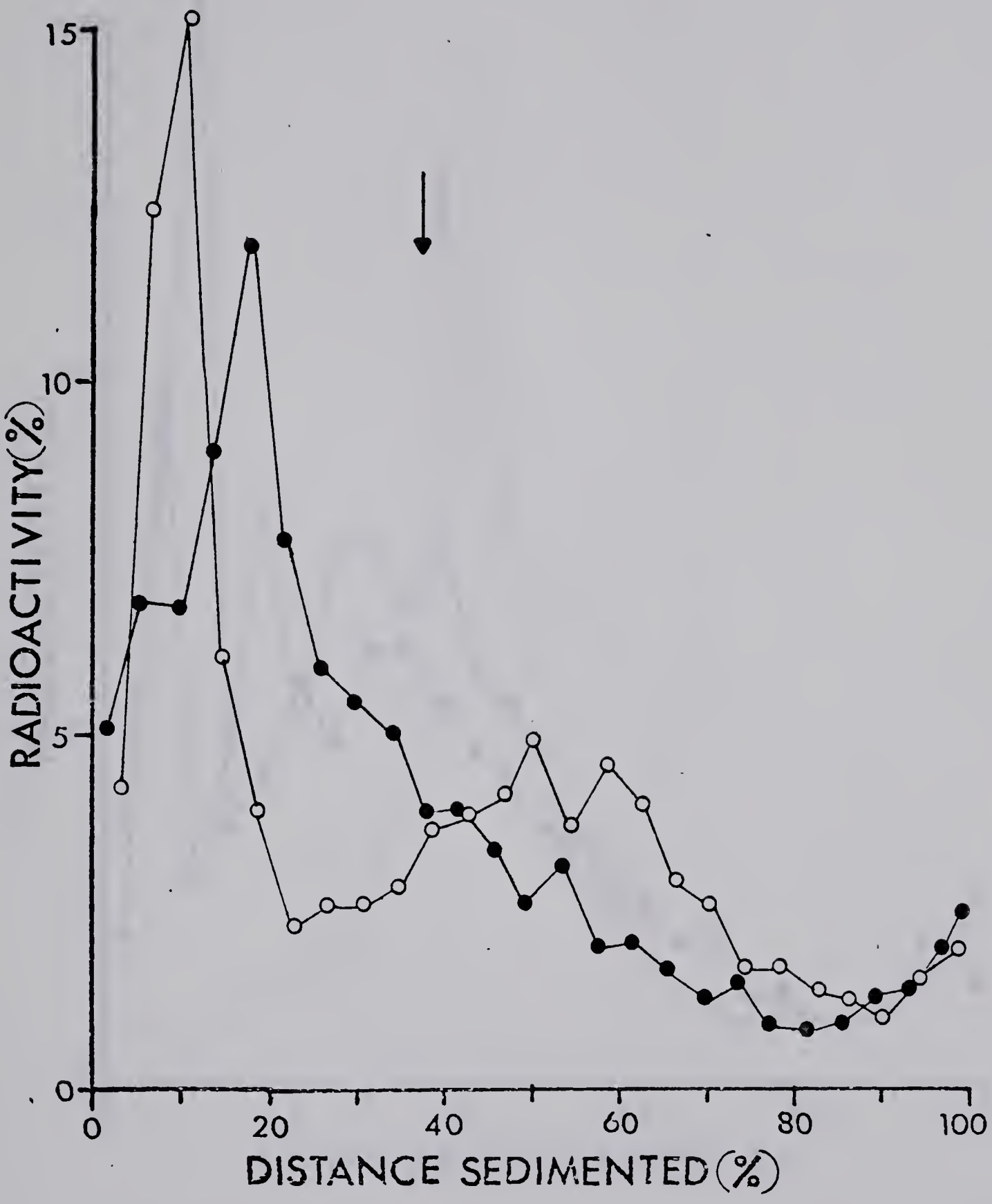


Fig. 11. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from diploid strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$, ρ^{-}) after 30 krads treated with AP endonuclease. The procedure was as described in figure 7. (O) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. Total ^{3}H cpm on each gradient was (O) 2382 cpm; (●) 2061 cpm.

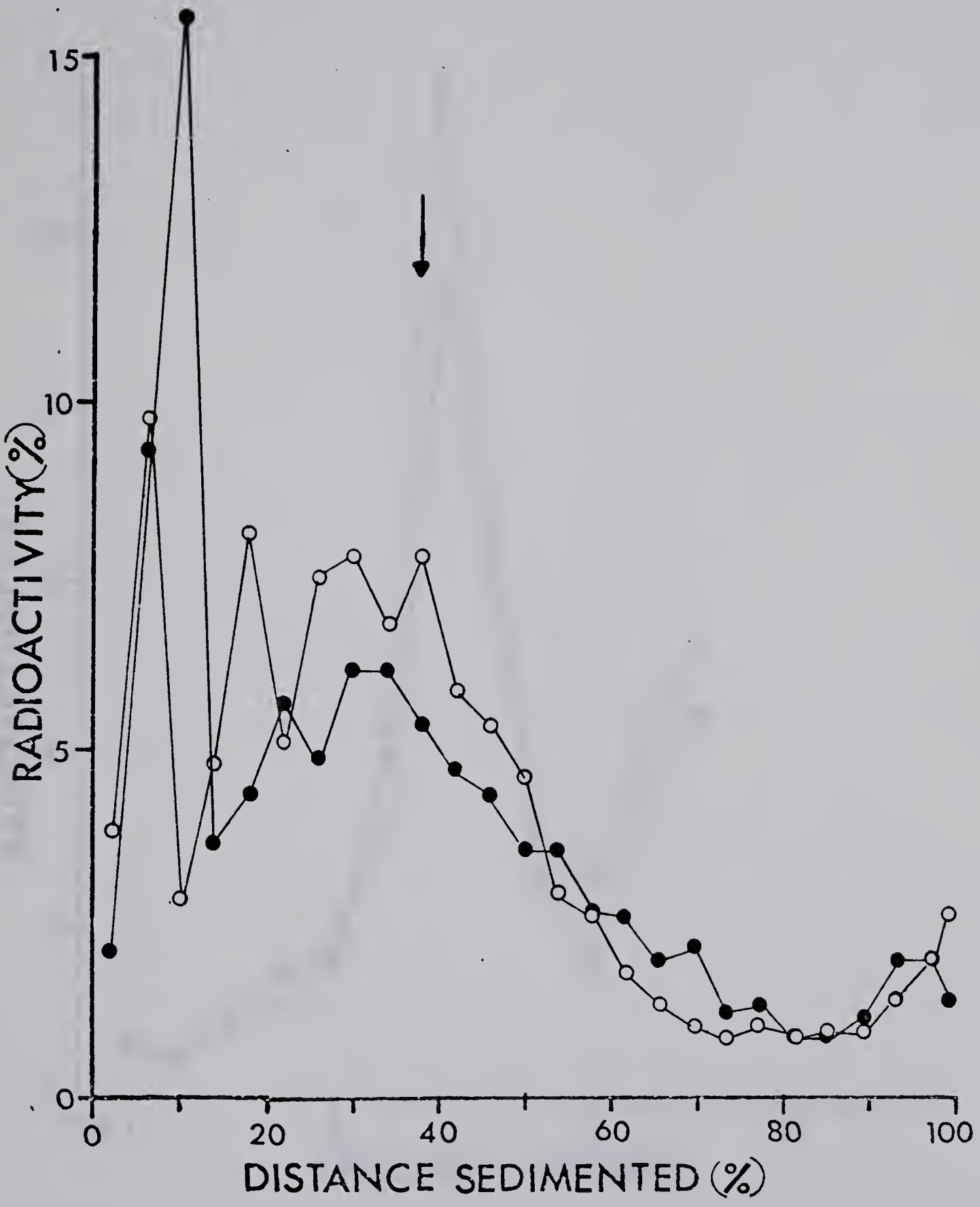


Fig. 12. Neutral sedimentation pattern of phenol purified, formamide denatured DNA from strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$, ρ^{-}) after 30 krads plus 4 hrs post-irradiation incubation treated with AP endonuclease. The procedure was as described in figure 7. (○) DNA not treated with AP endonuclease; (●) DNA treated with AP endonuclease. Total ^{3}H cpm on each gradient was (○) 1777 cpm; (●) 1597 cpm.

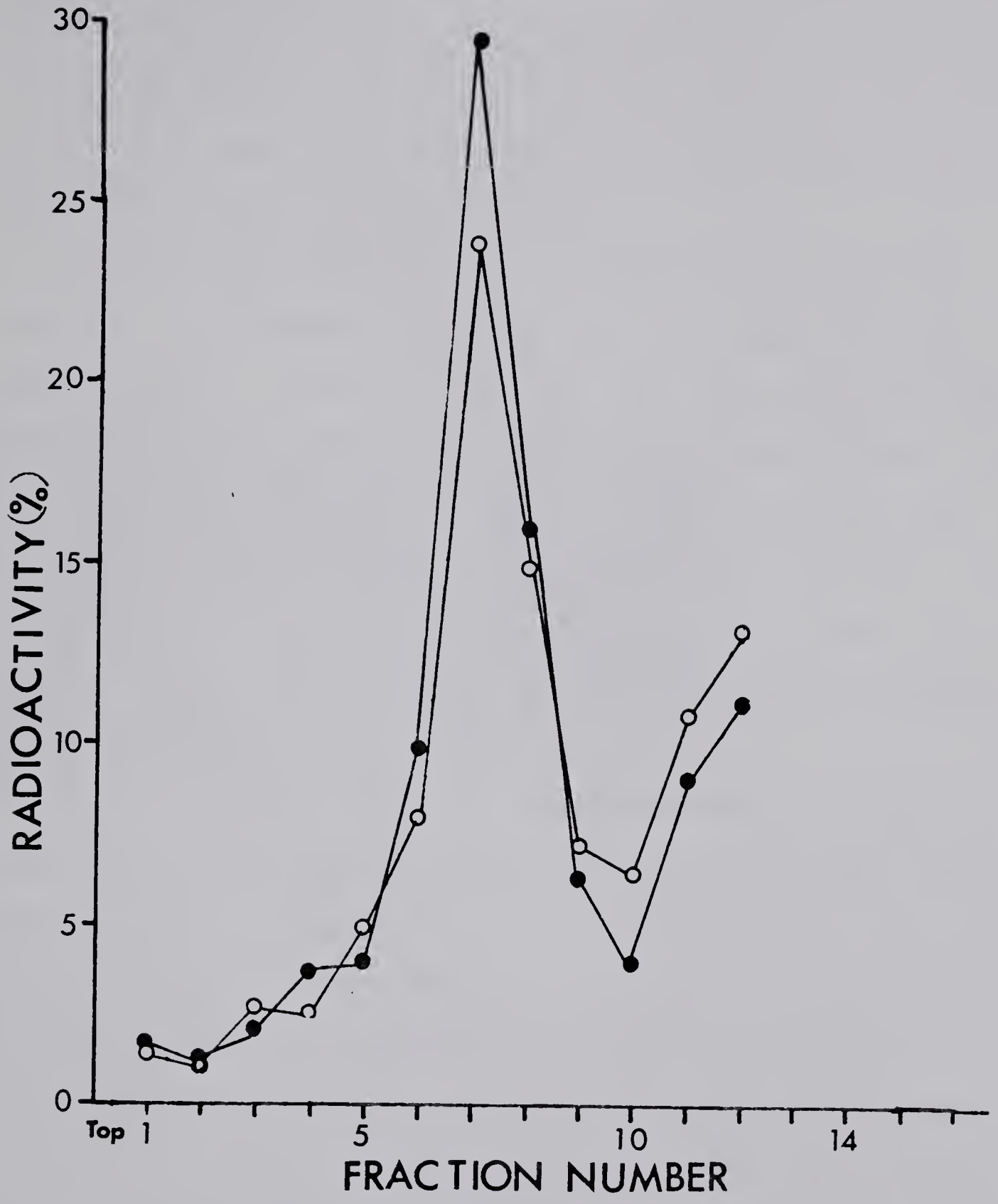


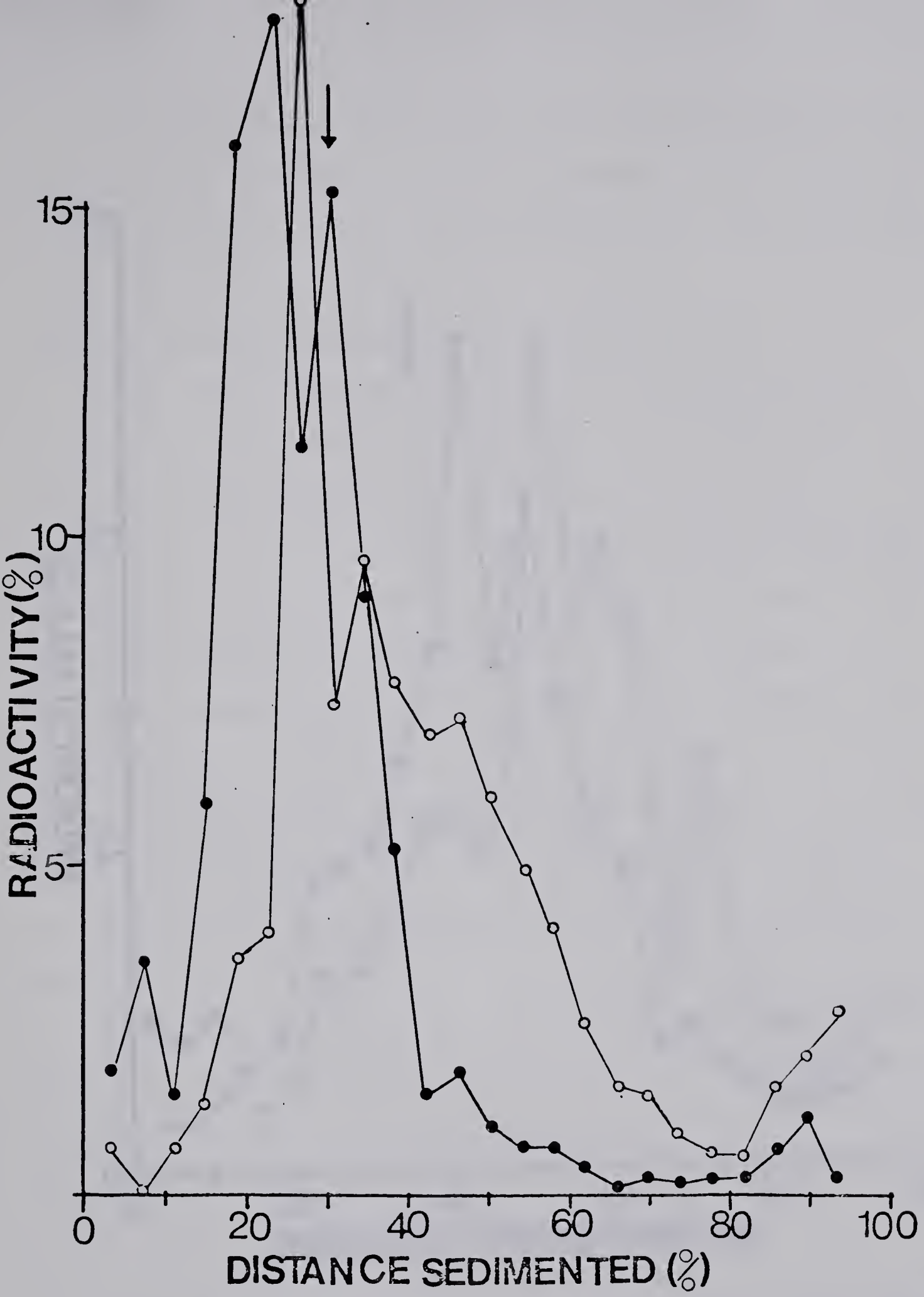
Fig. 13. Isopycnic centrifugation in CsCl of ^{3}H-uracil labelled material from *RAD*$^{+}$ and *rad51* homozygous ρ^{-} diploid strains. CsCl was dissolved in labelled cell lysates as described in Materials and Methods section. The mixture was centrifuged to equilibrium in a SW50L rotor at 33,000 rpm for 48 hrs. The CsCl gradient was fractionated by displacement with ethylene bromide and 0.4 ml fractions were taken. Radioactivity represents alkali stable acid precipitable counts of 10 µl samples of each fraction. (O) LA7 (*rad51/rad51*, ρ^{-}); (●) LA3 (*RAD*$^{+}$/*RAD*$^{+}$, ρ^{-}). Total cpm on each gradient was (O) 4090 cpm; (●) 5821 cpm.

Fig. 14. Neutral sedimentation pattern of CsCl density gradient purified, NaOH treated DNA from diploid strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$, ρ^{-}). The DNA solution was made 1 M with respect to NaOH and incubated at 25°C for 4 hours. The DNA sample not treated with NaOH had an equal volume of SSC added and incubated the same. Both DNA samples were denatured with formamide and formaldehyde as described in the Methods section. The denatured DNA was layered onto a 5-20% neutral sucrose gradient and spun at 35,000 rpm for 1.5 hrs in a SW50.1 rotor. (o) DNA not treated with NaOH; (●) NaOH treated DNA. The total ^{3}H cpm on each gradient was (o) 3899 cpm and (●) 1866 cpm.

are not prevalent in the DNA of unirradiated wild type or *rad51* strains and that AP sites can be repaired in both wild type and *rad51* strains. Because of shearing of DNA during phenol purification, it was not possible to determine whether the low molecular weight component is due to DNA single-strand breaks in *rad51* strains or some other type of alkali labile bond in the DNA besides AP sites.

A more gentle technique for the isolation of DNA is by CsCl isopycnic centrifugation. Figure 13 shows the results of a CsCl gradient used to purify yeast DNA. The major peak is yeast DNA with a density of 1.7 g/cc. The minor peak at the bottom of the tube is probably unhydrolysed RNA since RNA will pellet under the conditions used. The DNA from fractions 6 to 8 were pooled and dialysed against SSC. This DNA was treated with NaOH to test for the presence of alkali labile bonds and denatured with formamide and subjected to sucrose gradient centrifugation.

Figures 14 and 15 show the results of neutral gradients of DNA purified from CsCl gradients treated with NaOH and formamide denatured. The DNA isolated from wild type cells (fig. 14) was sheared more during preparation than DNA from *rad51-1* cells in this experiment. The DNA on these gradients has been shown to be single-stranded by W. J. Jachymczyk (pers. comm.) by the fact that there is no decrease in fluorescence with EtBr when heated, or when the pH is increased. High pH is known to cause strand breaks in DNA (Hill and Fangman, 1973), so that some breaks are expected after treatment with NaOH. DNA from a *rad51-1* strain is no more alkali labile than DNA from wild type cells. Attention should also be paid to the fact that the low molecular weight component, found on previous gradients with *rad51* strains is not seen. This would indicate that this component had a density in CsCl gradients different from DNA and therefore is not DNA.


RADIOACTIVITY(%)
15
10
5
0
20
40
60
80
100
DISTANCE SEDIMENTED (%)

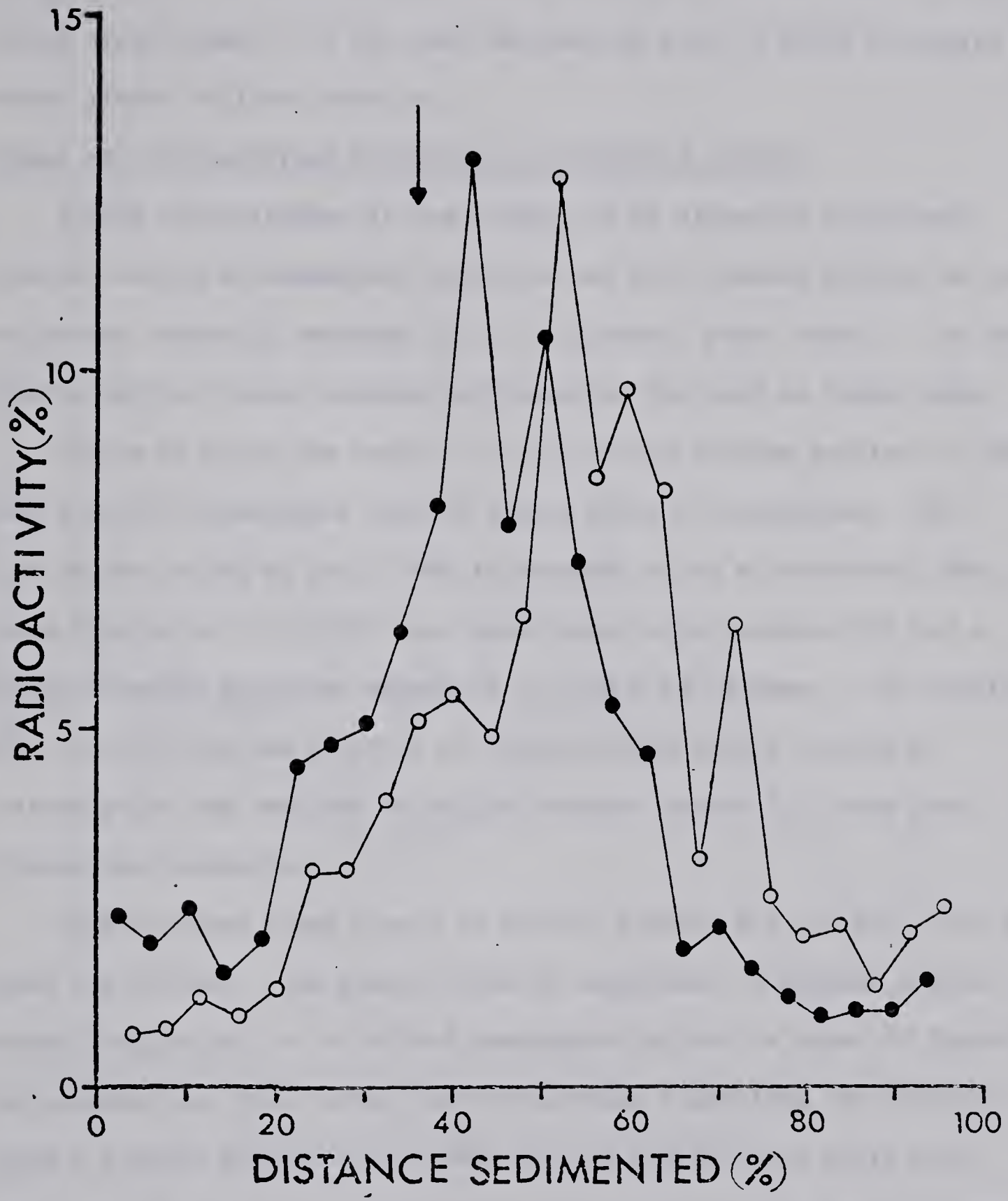


Fig. 15. Neutral sedimentation pattern of CsCl density gradient purified, NaOH treated DNA from diploid strain LA7 (*rad51/rad51*, ρ^-). The procedures were as described in figure 14. (o) DNA not treated with NaOH; (●) NaOH treated DNA. The total ^3H cpm on each gradient was (o) 7418 cpm and (●) 3717 cpm.

It seems clear from these results that the *RAD52* pathway is primarily involved in double-strand break repair and not AP site or single-strand break repair. In the next section the role of *RAD18* in repair of strand breaks will be looked at.

Single and Double-Strand Break Repair in *rad18-2* Strains

During these studies it was brought to my attention that yeast strains lacking mitochondrial functions may have reduced ability to carry out sister chromatid exchange (D. H. Williamson, pers. comm.). For this reason, petite strains lacking mitochondrial DNA were no longer used.

Figure 16 shows the results of an alkaline sucrose gradient of DNA from a *rad18-2* homozygous diploid strain after γ-irradiation. The peak in the region of the T7 DNA is presumed to be mitochondrial DNA, since Blamire *et al.* (1972) have shown yeast mitochondrial DNA has a double-stranded molecular weight of 2.5-5.0 x 10^7 daltons. The results show clearly that the majority of single-strand breaks induced by γ-irradiation are repaired by *rad18-2* strains within 3.5 hours post-irradiation incubation.

Double-strand break repair in *rad18-2* strains did not give such a clear cut picture. The results from an experiment to measure double-strand break repair in a *rad18-2* homozygous diploid is shown in figure 17. The results show that during post-irradiation incubation the DNA from *rad18-2* strains becomes more broken up than the DNA from cells just receiving 30 krads of γ-irradiation. For comparison, the results from a wild type strain under similar conditions are shown in figure 18. The wild type strain shows a repair of double-strand breaks with post-irradiation incubation. The DNA from cells which were incubated after irradiation does not show any increase in breakage compared to the DNA from just irradiated cells. Similar results for a wild type petite strain can be seen in figure 3.

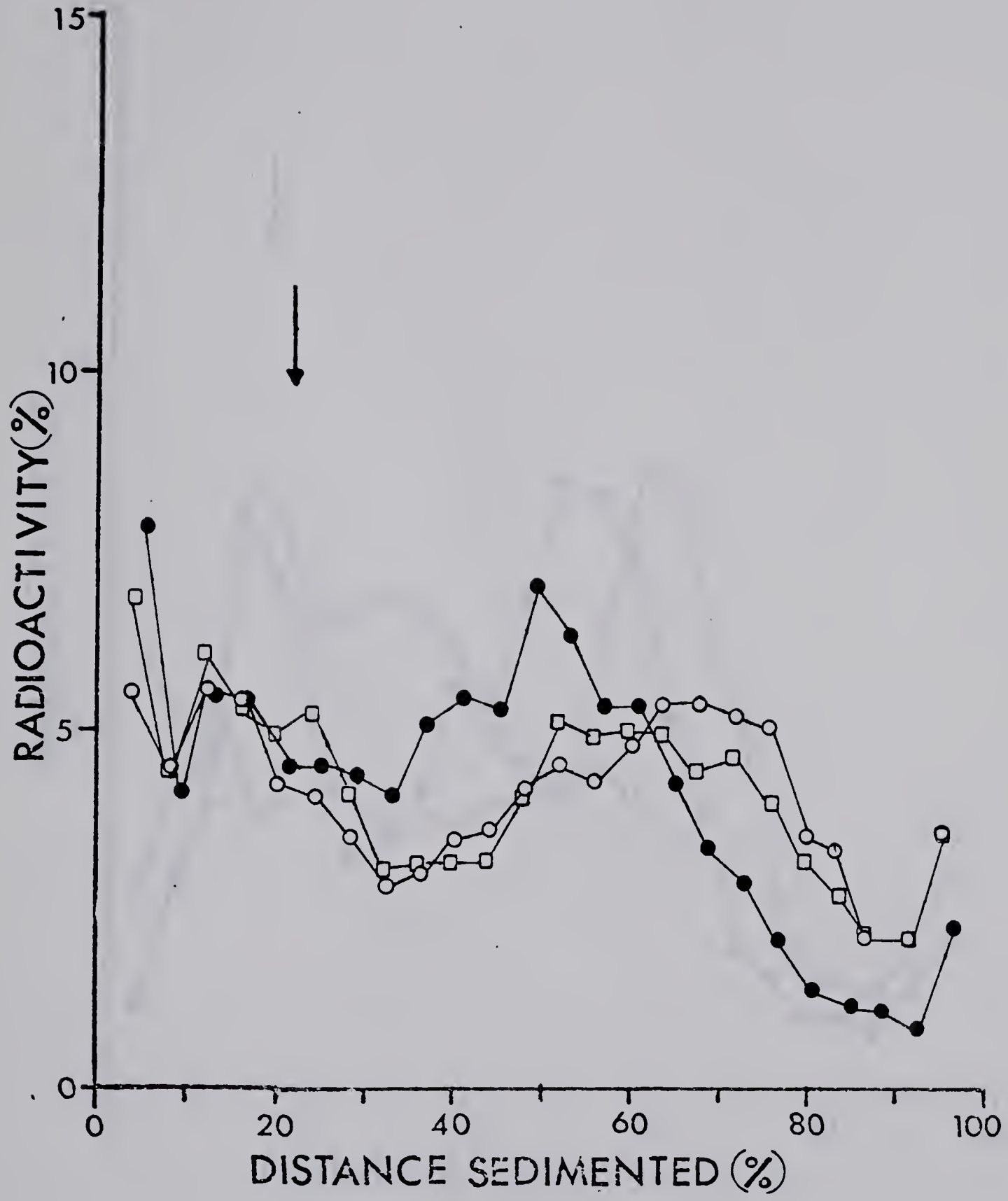


Fig. 16. Alkaline sedimentation pattern of DNA from diploid strain LA4 (*rad18-2/rad18-2*) after 20 krads. Irradiation and post-irradiation procedures are as given in figure 3. Spheroplasts were layered onto a 15-30% alkaline sucrose gradient. Centrifugation was at 11,000 rpm for 26 hrs in a SW50.1 rotor. (○) 0 rads; (●) 20 krads; (□) 20 krads + 3.5 hrs post-irradiation incubation in YEPD. Total ^{3}H counts on each gradient was (○) 8106 cpm; (●) 9040 cpm; (□) 6243 cpm.

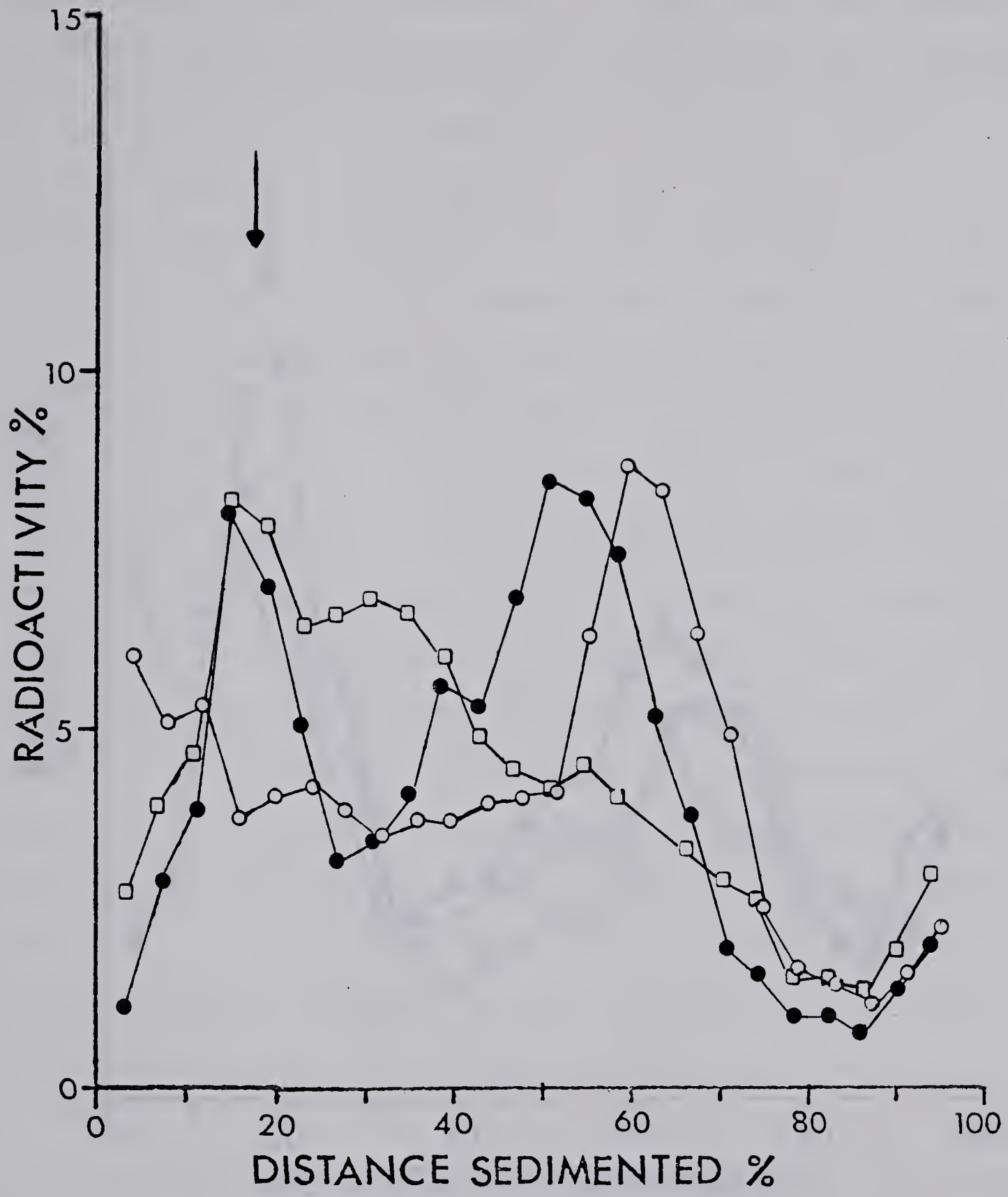


Fig. 17. Neutral sedimentation pattern of DNA from diploid strain LA4 (*rad18-2/rad18-2*) after 30 krads. Irradiation and post-irradiation procedures are the same as given in Fig. 3 except incubation time. Spheroplasts were layered onto a 15-30% neutral sucrose gradient. Centrifugation was at 12,000 rpm for 26 hrs in a SW50L rotor. (○) no irradiation; (●) 30 krads only; (□) 30 krads + 3.5 hrs post-irradiation incubation in YEPD. The total ^{3}H cpm on each gradient is (○) 8270 cpm; (●) 7547 cpm; (□) 6972 cpm.

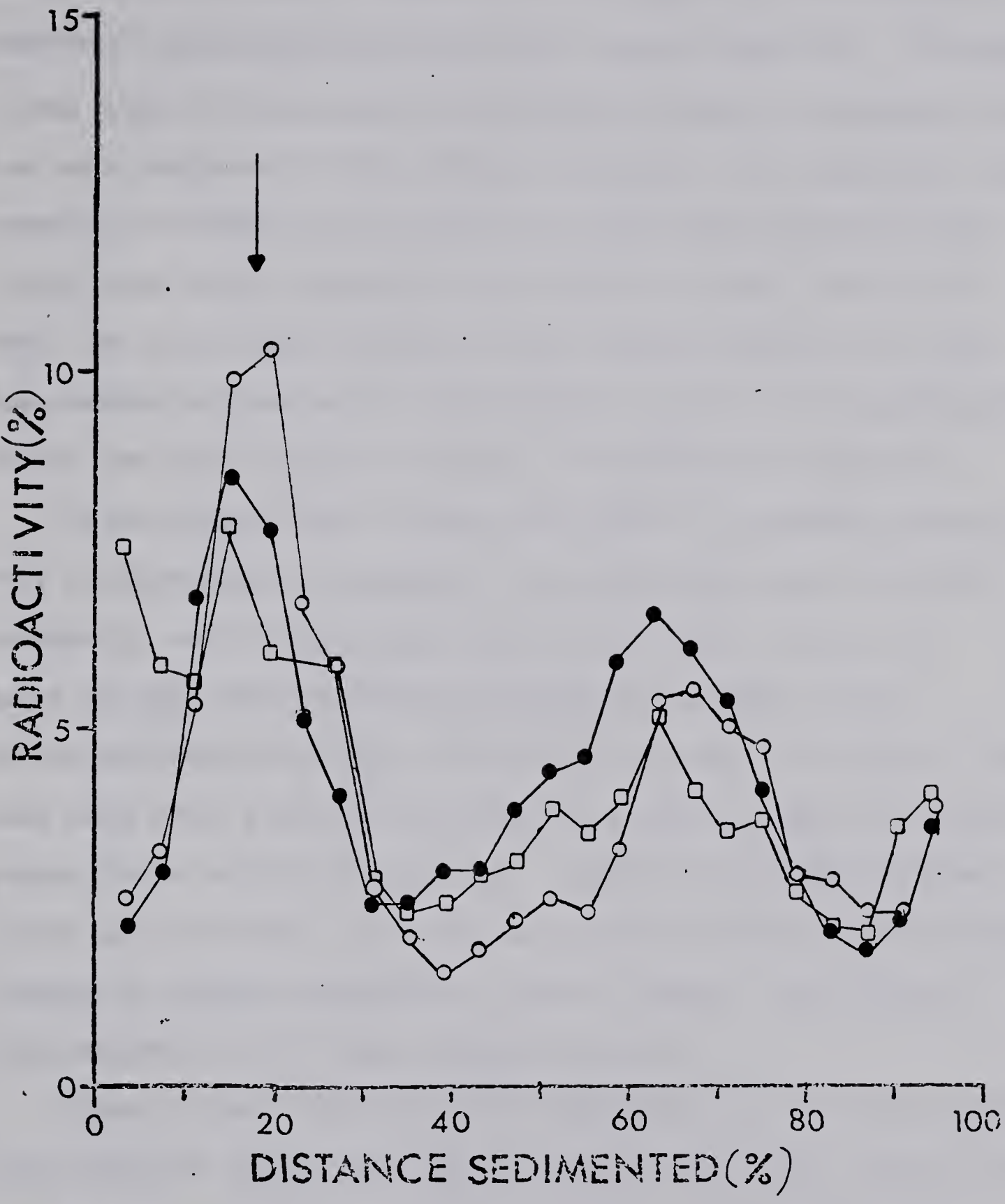


Fig. 18. Neutral sedimentation pattern of DNA from a wild type diploid strain LA3 (*RAD*$^+$/*RAD*$^+$) after 30 krads. Irradiation and post-irradiation procedures are the same as given in figure 3. Spheroplasts were layered onto a 15-30% neutral sucrose gradient. Centrifugation was at 11,000 rpm for 26 hrs in a SW50.1 rotor. (○) 0 rads; (●) 30 krads; (□) 30 krads + 3.5 hrs post-irradiation incubation in YEPD. The total ^3H cpm on each gradient was (○) 4142 cpm; (●) 4720 cpm; (□) 3980 cpm.

The break-down of DNA seen on neutral gradients (figure 17) in *rad18-2* strains tended to contradict the results seen in the previous experiment measuring single-strand break repair (figure 16). If double-strand breaks are occurring during the post-irradiation incubation period, how could single-strand break repair be detected? The possibility that a revertant of *rad18-2* had occurred in the experiment measuring single-strand break repair (figure 16) can be ruled out since, from a spot test, the culture used was seen to have remained sensitive to γ-rays. One possible explanation for the difference between the two experiments is the dose used, 30 krads in figure 17 and 20 krads in figure 16.

To see whether *rad18-2* strains are capable of repairing breaks as well as experiencing a breakdown of their DNA during post irradiation incubation, the following experiment was performed. A dose of 20 krads was used and both alkaline and neutral gradients were run at the same time using cells from the same cultures. In addition, samples were taken after 8 hours post-irradiation incubation, made into spheroplasts, frozen and run the next day. A sample of unirradiated spheroplasts was also frozen. It showed no break-up of DNA caused by freezing compared to unfrozen spheroplasts (data not shown). The results of this experiment can be seen in figures 19 and 20.

Figure 19 shows that the *rad18-2* homozygous diploid strain used has some ability to repair γ-ray induced double-strand breaks. This can be seen as a movement back to the high molecular weight region (40-55% distance sedimented) from the 20 krad curve, without any concommitant increase in low molecular weight material. The amount of repair seen is not as extensive as seen in wild type cells. DNA from cells incubated for 8 hours after irradiation shows a decrease in molecular weight compared to the DNA from cells irradiated without incubation or with 3.5

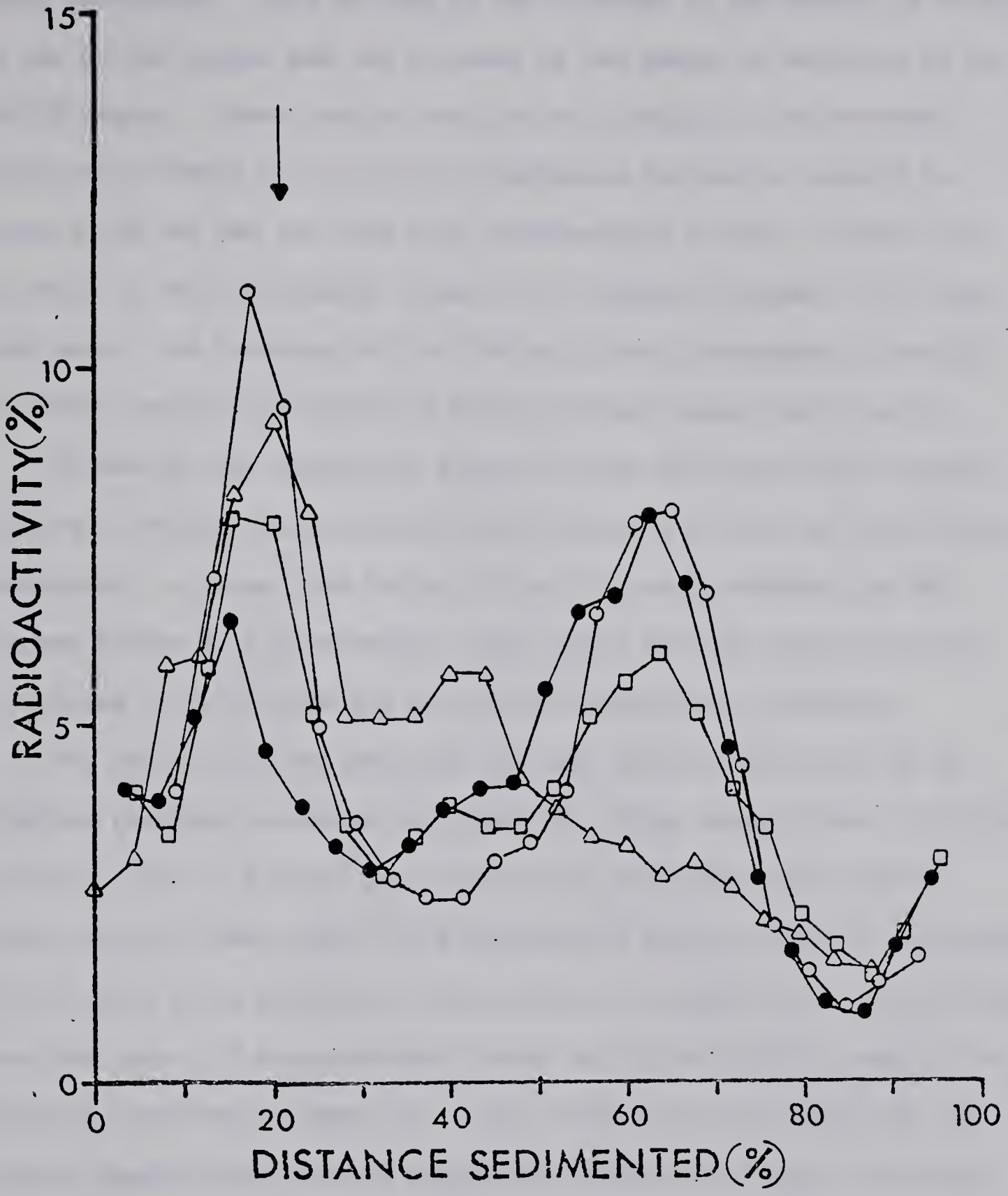


Fig. 19. Neutral sedimentation pattern of DNA from diploid strain LA4 (*rad18-2/rad18-2*) after 20 krads. Irradiation and post-irradiation procedures are as given in figure 3 except for dose and time. Spheroplasts were layered onto a 15-30% neutral sucrose gradient. Centrifugation was at 11,000 rpm for 26 hrs in a SW50.1 rotor. The 8 hr post irradiation incubation sample was run at 12,000 rpm for 26 hrs in a SW50L rotor. The profile for the 8 hr sample was normalized to the T7 DNA peak of the other samples. (○) 0 rads; (●) 20 krads; (□) 20 krads + 3.5 hrs post-irradiation incubation in YEPD; (△) 20 krads + 8 hrs post irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (○) 5469 cpm; (●) 7169 cpm; (□) 6220 cpm; (△) 5342 cpm.

hours incubation. This is seen by the increase in the amount of material in the 25-50% region and the decrease in the amount of material in the 50-75% region. These results confirm the findings in the previous experiment (figure 17) that post-irradiation incubation results in break up of the DNA (in this case double-strand breaks). Repair was not seen in this experiment (figure 17), probably because of the high dose used. The breakdown of the DNA with post-irradiation incubation may have obscured any repair of double-strand breaks that occurred.

To sum up, the results in figure 19 show that the *rad18-2* mutant appears to repair double-strand breaks within 3.5 hours of post-irradiation incubation. At some time during 3.5 to 8 hours incubation the DNA becomes broken to a size smaller than either the DNA from cells just irradiated or cells with 3.5 hours post-irradiation incubation.

The results for the DNA from the same cells as above but on an alkaline gradient are shown in figure 20. These results show a similar pattern. After 3.5 hours post-irradiation incubation this *rad18-2* strain clearly shows repair of single-strand breaks caused by γ-irradiation. This is seen by an increase in high molecular weight DNA. This confirms that the repair of single-strand breaks in *rad18-2* strains seen in the previous experiment (figure 16). With continued incubation, the DNA becomes smaller than the DNA from cells incubated for only 3.5 hours.

It may be argued that the break-up of DNA with post irradiation incubation in *rad18-2* strains may be an artifact of using a high radiation dose and low survival. To obtain information on this point a *rad18-2* strain was irradiated with a low dose of γ-rays (10 krads, 8-10% survival) and placed in post-irradiation incubation medium for 3.5 and 8 hours. For comparison a wild type strain was irradiated at this dose and incubated for 8 hours. The results of this experiment can be seen in figures 21 and 22.

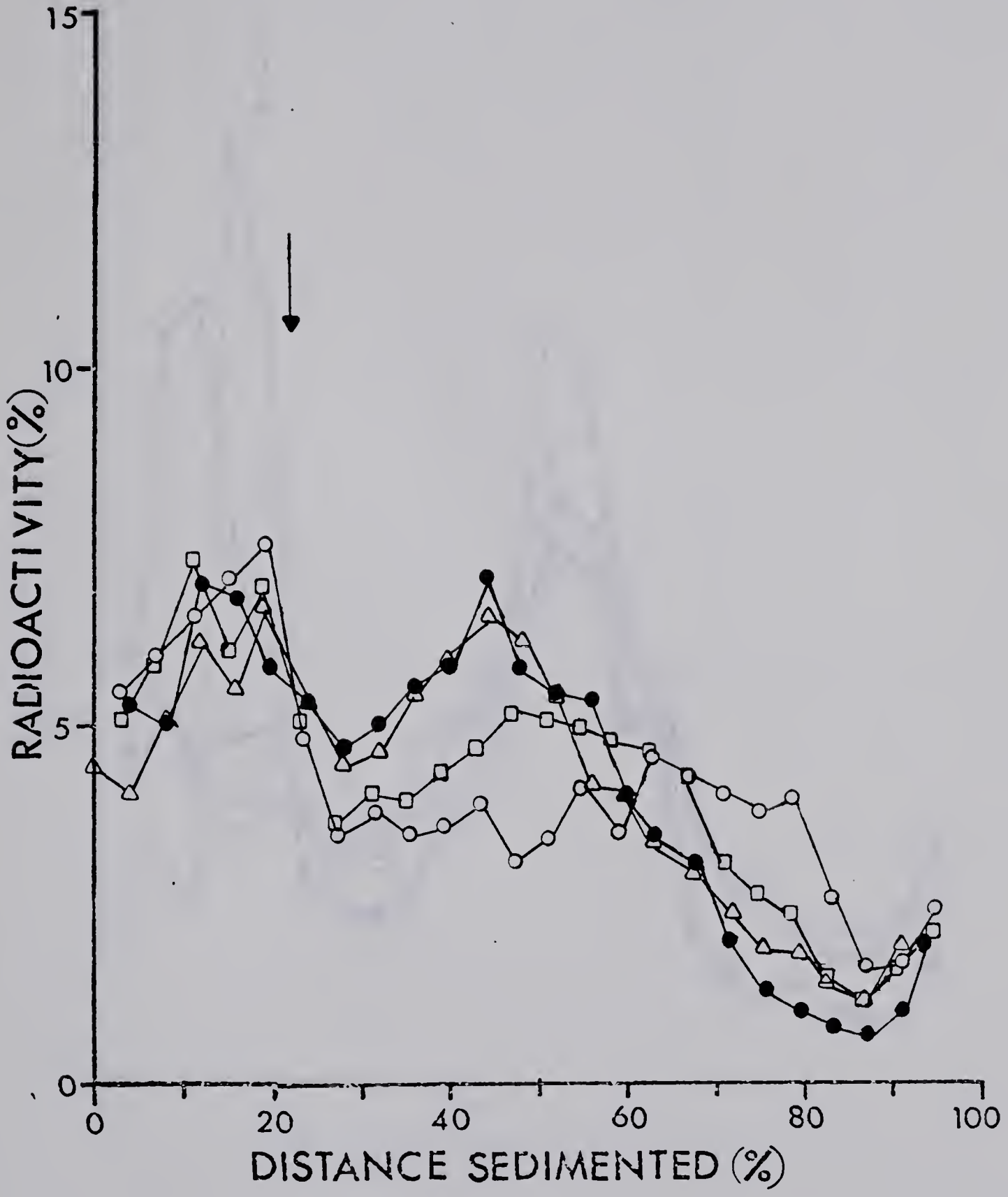


Fig. 20. Alkaline sedimentation pattern of DNA from diploid strain LA4 (*rad18-2/rad18-2*) after 20 krads. The spheroplasts used on these gradients are from the same samples as used in figure 19. The procedures are as given in figure 19 except that spheroplasts were layered onto 15-30% alkaline sucrose gradients. (○) 0 rads; (●) 20 krads; (□) 20 krads + 3.5 hrs post-irradiation incubation in YEPD; (△) 20 krads + 8 hrs post irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (○) 6804 cpm; (●) 9201 cpm; (□) 8217 cpm; (△) 8349 cpm.

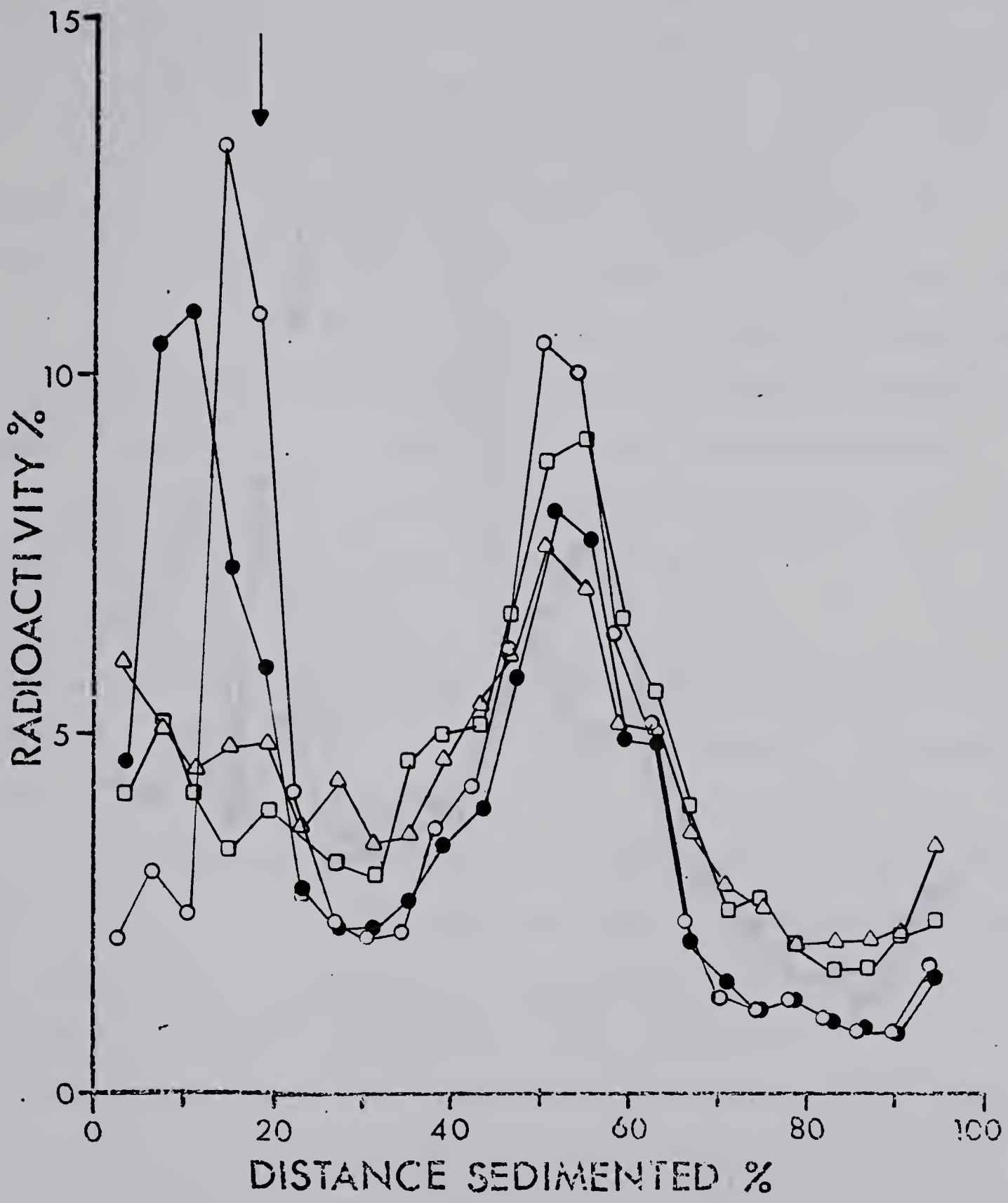


Fig. 21. Neutral sedimentation pattern of DNA from a diploid strain LA4 (*rad18-2/rad18-2*) after 10 krads. Irradiation and post-irradiation procedures are as given in figure 3 except for dose and time. Spheroplasts were layered onto a 15-30% neutral sucrose gradient. Centrifugation was at 11,000 rpm for 26 hrs in a SW50.1 rotor. (○) 0 rads; (●) 10 krads; (□) 10 krads + 3.5 hrs post-irradiation incubation in YEPD; (△) 10 krads + 8 hrs post irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (○) 4681 cpm; (●) 5394 cpm; (□) 3797 cpm; (△) 3497 cpm.

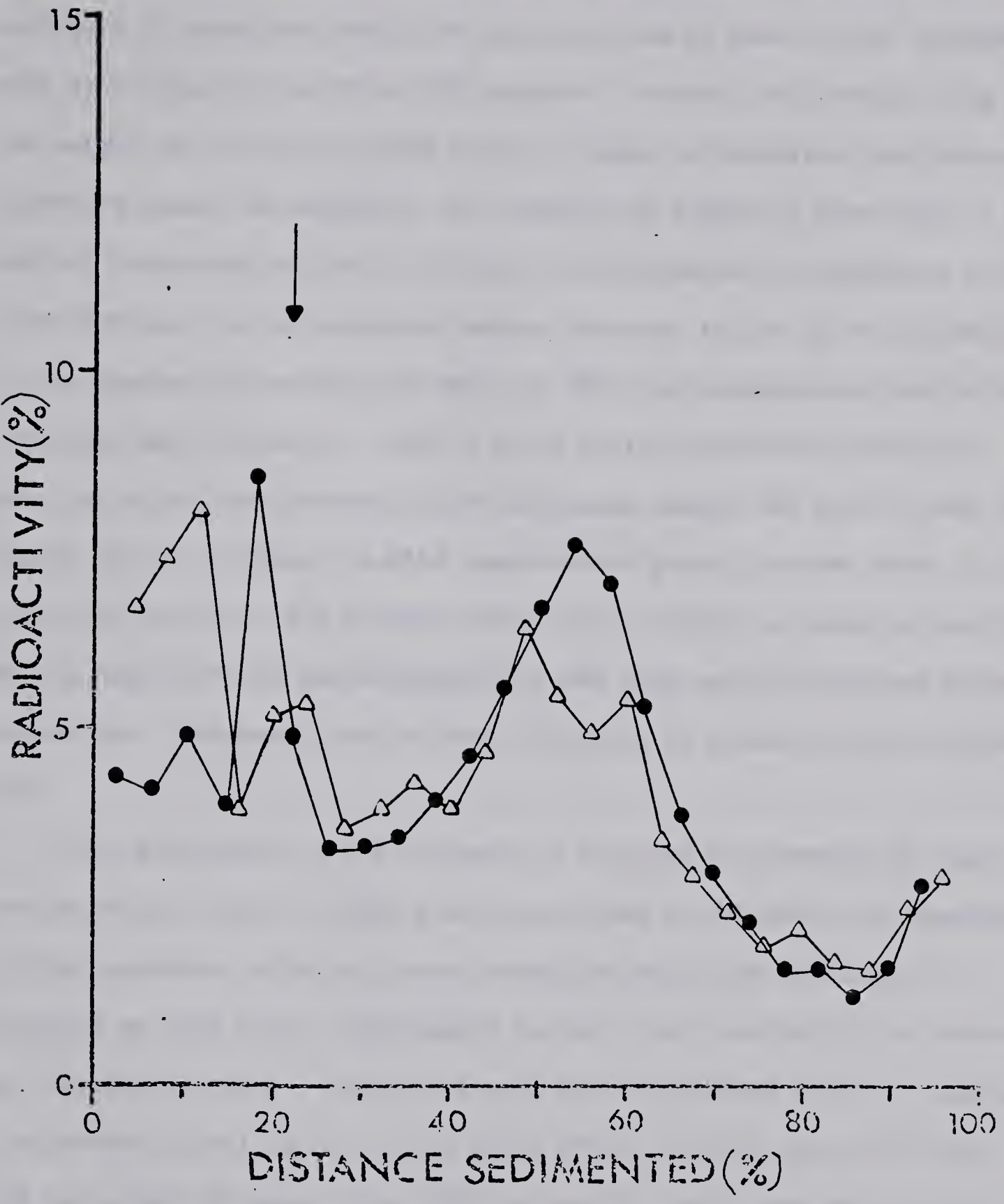


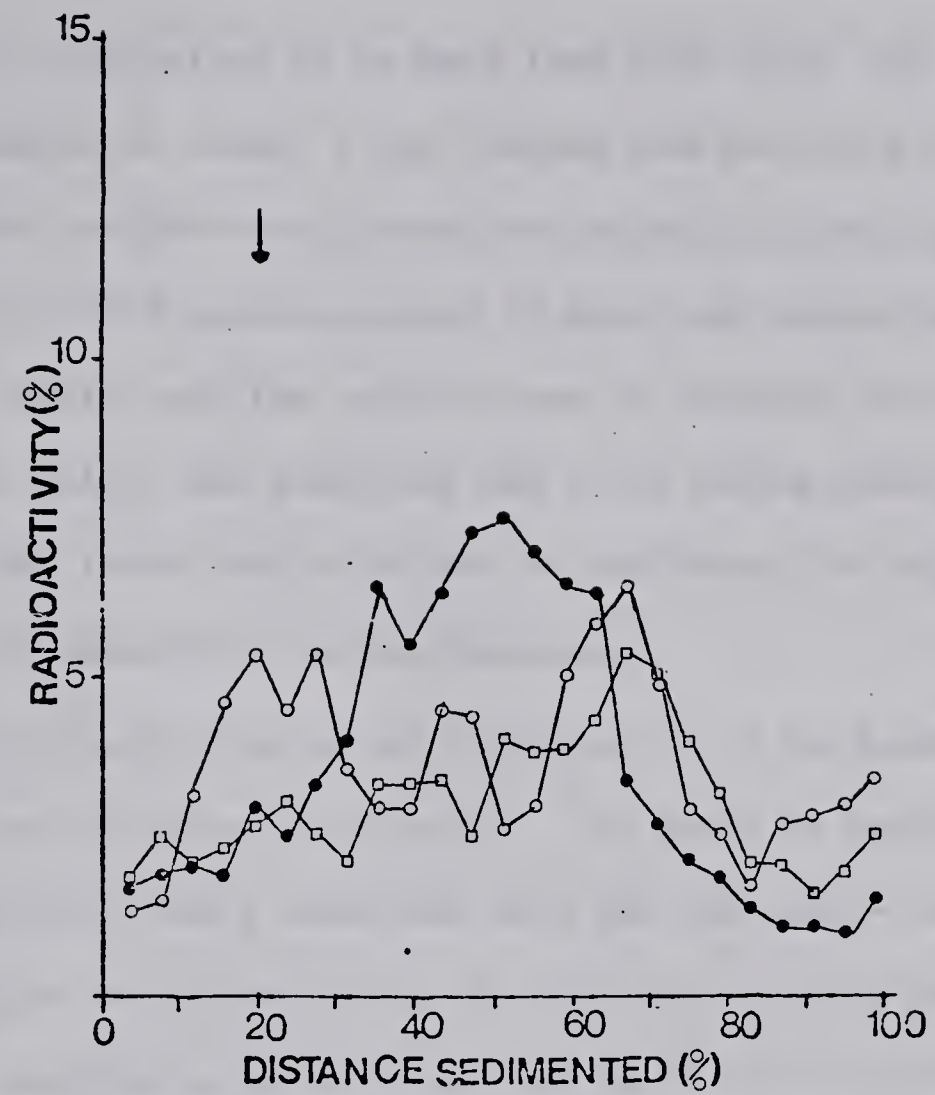
Fig. 22. Neutral sedimentation pattern of DNA from wild type diploid strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$) after 10 krads. Procedures were the same as given in figure 21. (●) 10 krads; (Δ) 10 krads + 8 hrs post-irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (●) 1993 cpm; (Δ) 2385 cpm.

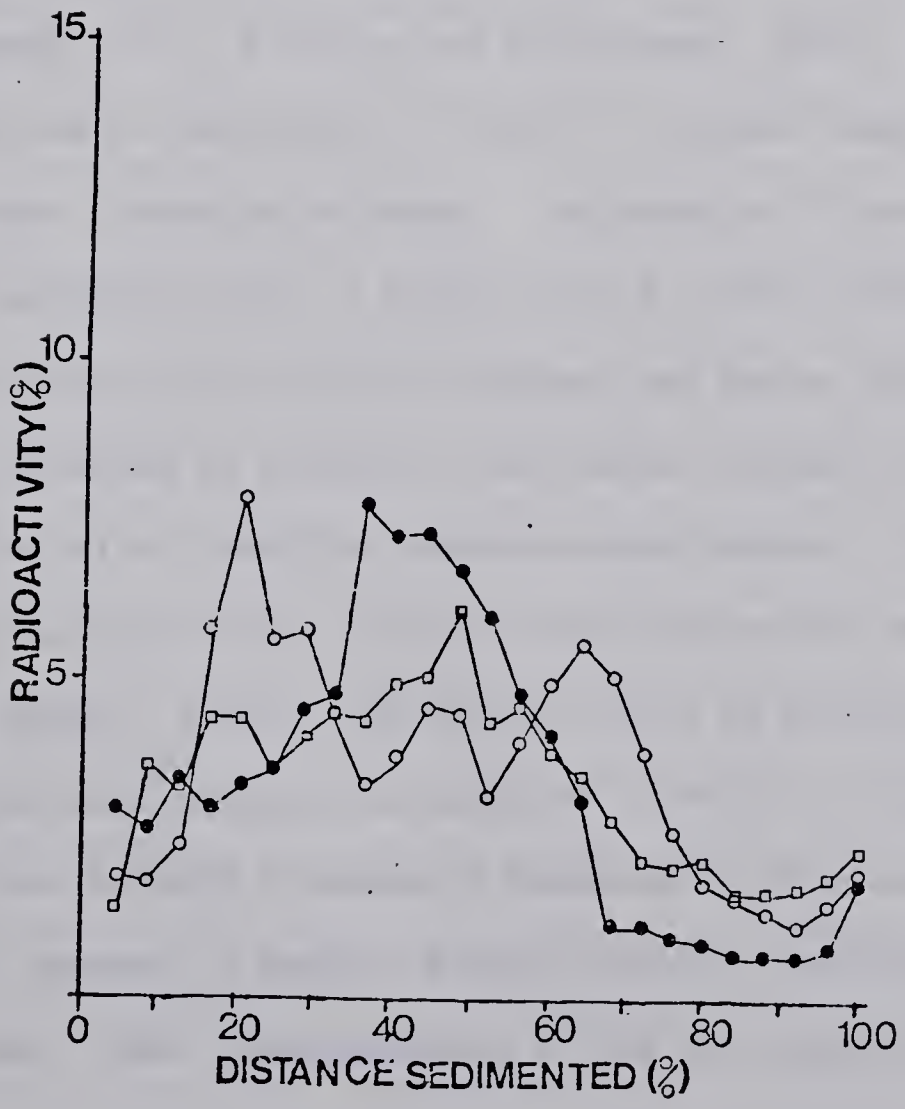
Double-strand breakage induced by 10 krads γ-rays is less than can be detected by the neutral sucrose gradient technique. This can be seen in figure 21 where the zero dose curve and the 10 krad curves coincide with each other in the 30 to 80% regions; the only difference being in the height of the peak in this region. Repair of double-strand breaks therefore cannot be detected. The results in figure 21 show that in a *rad18-2* homozygous diploid, 3.5 hours post-irradiation incubation results in an increase in low molecular weight material in the 22 to 42% region of the gradient above what is seen for DNA from unirradiated and cells receiving only 10 krads. After 8 hours post irradiation incubation there is a further increase in low molecular weight DNA seen in the 25% region of the gradient. A wild type diploid given the same dose of 10 krads and incubated for 8 hours shows only a slight increase in the 28 to 36% region of the curve compared to DNA from cells irradiated without incubation. The small peak in the 24% region is probably mitochondrial DNA.

This experiment is not adequate to rule out the possibility that the break-up of DNA in *rad18-2* strains is due to a generalized degradation of DNA associated with cell death since the wild type has higher survival at this dose. Experiments to test this possibility are shown in figures 23a and b. These data were kindly provided by W. J. Jachymczyk. The survival level for wild type cells after 75 krads (fig. 23a) was 26% and after 100 krads (fig. 23b) it was 3%. Wild type cells irradiated at a dose of 75 krads (fig. 23a) can repair double-strand breaks with 8 hours post-irradiation incubation. After a dose of 100 krads wild type cells still repair double-strand breaks but to a lesser extent (fig. 23b). In both experiments there was no increase in low molecular weight material after 8 hours post-irradiation incubation

compared to the size of DNA from cells irradiated without incubation. Resnick and Martin (1976) have also shown in a wild type diploid yeast that six hours post-irradiation incubation after 75 krads (10% survival) does not result in a significant increase in low molecular weight DNA compared to the DNA from irradiated cells without incubation. These results are to be contrasted with the findings of *rad18-2* for 8-10% survival (fig. 21) and 2% survival (fig. 19) where 8 hours post-irradiation incubation leaves the DNA smaller than the size of DNA from cells receiving only irradiation. Other data indicate that the breakdown of DNA in *rad18-2* strains is not due to cell death are seen in figures 5 and 17. The *rad51* homozygous diploids at 30 krads have approximately 0.1% survival whereas *rad18-2* strains have 1% survival (figure 1). The *rad51-1* cells at the same dose and post incubation time as *rad18-2* cells do not show a break-up of their DNA during the post irradiation incubation period (figure 5). Strains carrying *rad18-2* do show a decrease in molecular weight with post irradiation incubation (figure 17) compared to controls shown. The above data seem to indicate that the break-up of DNA in *rad18-2* strains does not reflect a generalized degradation of DNA associated with cell death but reflects something intrinsic to *rad18-2* strains.

Fig. 23a and b. Neutral sedimentation pattern of DNA from diploid strain LA3 (*RAD*$^{+}$/*RAD*$^{+}$) after (a) 70 krads and (b) 100 krads. Irradiation and post-irradiation procedures are as given in fig. 3 except for dose and incubation time. Spheroplasts were layered onto a 15-30% neutral sucrose gradient. Centrifugation was at 11,000 rpm for 26 hrs in a SW50.1 rotor. (a) (o) No irradiation; (●) 70 krads only; (□) 70 krads + 8 hrs post-irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (o) 10,027 cpm; (●) 12,700 cpm and (□) 23,339 cpm. (b) (o) No irradiation; (●) 100 krads only; (□) 100 krads + 8 hrs post-irradiation incubation in YEPD. The total ^{3}H cpm on each gradient was (o) 9556 cpm; (●) 7923 cpm and (□) 24,237 cpm.


a
15
10
5
RADIOACTIVITY(%)
0
20
40
60
80
100
DISTANCE SEDIMENTED (%)


b
15
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RADIOACTIVITY(%)
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DISCUSSION

The major conclusions to be made from this study are that *rad51* strains are unable to repair γ-ray induced double-strand breaks, but they can repair single-strand breaks and apurinic sites in DNA. On the other hand, *rad18* strains appear to have both double and single-strand break repair, and the results seem to indicate that the molecular weight of both native and denatured DNA falls during post-irradiation incubation. How these results relate to the theory of repair mechanisms and pathways in yeast will now be discussed.

The first point of potential relevance is to be found in the survival curves presented in figure 1. The point to notice is that the *rad18-2* diploid has a resistant tail and the *rad51-1* strain does not. In haploid wild type yeast, the resistant tail is known to be due to cells undergoing DNA synthesis or in G2 of the cell cycle (Bird and Manney, 1974; Brunborg and Williamson, 1978). The resistance is presumably due to the ability of cells to repair double-strand breaks by sister chromatid exchange. Log phase wild type diploids also show a resistant tail, at high doses of γ-rays (60-100 krads) which can be removed with caffeine (Hannan and Nasim, 1977). Again this tail is believed to indicate that sister chromatid recombination increases survival by repairing double-strand breaks. Also in the same paper it was shown that a *rad52* haploid (known not to have double-strand break repair, Resnick and Martin, 1976; Ho and Mortimer, 1975) showed no sensitization to caffeine after U.V. Together these facts imply that a *rad18-2* strain is expected to show double-strand break repair, whereas, a *rad51-1* strain with its exponential decline in survival cannot. This interpretation of the tail region may be invalid

if suppressors of the *rad18-2* phenotype were in the background of the strain. This objection would have to be checked out by seeing if *rad18* strains existed without a tail on their survival curves.

The results with *rad51-1* homozygous diploid strains show that they have the ability to repair γ-ray induced apurinic sites and single-strand breaks but not double-strand breaks. The slight increase in molecular weight seen on the neutral gradient (figure 4a) with post-irradiation incubation may reflect single-strand break repair that looks like a double-strand break on a neutral gradient but *in vivo* does not result in a double-strand break.

The low molecular weight material seen in both neutral and alkaline gradients of *rad51-1* strains is probably not DNA since purification of DNA by CsCl density gradients resulted in the disappearance of this component. Since ^{3}H-uracil was used as the label, this component is probably RNA. A possible reason for its excess in *rad51-1* strains is their larger size compared to wild type cells as seen in the light microscope. The increase in volume of the cytoplasm could reflect an increase in an RNA (protein) component that is resistant to alkaline hydrolysis. Hatzfeld (1973) reported 1-3% of RNA in yeast is made up of 2 or 3'-O methyl-ribose which is more resistant to alkali. This explanation may also explain K. Ho's results with *rad52* (Ho, 1975b).

The finding that *rad51-1* cells cannot repair double-strand breaks lends support to the validity of forming epistasis groups from genetic experiments. The mutants *rad52-1*, *rad6-1* and *rad51-1* are all within the same epistasis group for X-ray survival (Game and Mortimer, 1974). All of these mutants have been shown to be defective in double-strand break repair (Resnick and Martin 1976; Ho and Mortimer, 1975; Chlebowicz and Jachymczyk, 1978; this study). Another biochemical support for the value

of epistatic grouping of repair mutants is seen in the *rad3* pathway for U.V. dimer excision (Prakash, 1977a,b).

The results with the AP endonuclease show that apurinic sites are not detectable at the spontaneous level in wild type and *rad51-1* strains. It has been shown that depurination of DNA under physiological conditions is quite significant (Lindahl and Nyberg, 1972). Any mutant defective in AP endonuclease activity would be expected to show alkali labile bonds (AP sites). The fact that yeast have at least two apurinic endonucleases (Armel and Wallace, 1978) may mean that loss of one endonuclease activity can be compensated by the other and may obscure the phenotype.

The finding that *rad51-1* cells can repair AP sites implies that the *RAD51* function, which is needed for double-strand break repair, is not needed for repair of apurinic sites.

One of the assumptions made in interpreting a synergistic interaction between *rad* mutants is that the gene products are competing for the same lesion (Brendel and Haynes, 1973; Game and Cox, 1973; Cox and Game, 1974). Members of the *rad52* epistasis group show a synergistic interaction with *rad18* (Game and Mortimer, 1974). Resnick and Martin (1976) calculated, based on the efficiency of double-strand break repair and survival curves, that there are approximately 1.5 double strand breaks per lethal event in a *rad52 rad18* double mutant. Whereas a *rad52* single mutant needs 2.2 breaks per lethal event, *rad18* needs 20 and wild type cells 14.8 to 38 (depending on the region of the survival curve used). These results imply that *rad18-1* is involved in a minor way with double-strand break repair.

When comparing the results between different alleles of the same

rad locus, I am making the assumption they both represent a loss of function and not altered function.

The results presented in this study with *rad18-2* homozygous diploids offers an alternative explanation for synergistic interactions. Diploid strains homozygous for *rad18-2* appear to repair double-strand breaks. The reason the repair does not appear as great as seen in wild type cells may be due to the breakage of DNA that is occurring in *rad18-2* cells and which eventually predominates. So the synergistic effect caused by *rad18* with the *rad52* epistasis group may be due to *rad18* causing an increase in double-strand breaks, which overload the *rad52* repair pathway, leading to an increase in lethality caused by double-strand breaks. The double-strand breaks appear to be formed through the formation of closely occurring single-strand breaks since single-strand breaks occurring with incubation are in excess of double-strand breaks. An analogous finding was seen in a *pol A1* mutant of *E. coli* (Bonura, *et al.*, 1975a) where the rate of DNA double-strand break formation was found to be higher than in wild type cells after γ-irradiation. By cooling the cells, the rate in *pol A1* (DNA polymerase I) cells was decreased, but remained the same in wild type cells, implying an enzymatic cause of double-strand breaks.

If the breaks formed in a *rad18-2* strain are not due to a generalized breakdown of DNA with death, how is it that *rad18-2* strains form breaks in their DNA after seemingly repairing the initial scissions caused by gamma-rays? One possible explanation is that *rad18-2* strains fail to fill in gaps created during repair of either base damage or disruptions in DNA synthesis across the site of a lesion. Evidence for *rad18-2* being involved in base excision comes from the fact that it is sensitive to the anti-folate drug trimethoprim (Game *et al.*, 1975). Trimethoprim

inhibits the synthesis of tetrahydrofolate by the enzyme dihydrofolate reductase. Tetrahydrofolate is needed for the *de novo* synthesis of thymidylate (TMP) from deoxyuridine monophosphate (dUMP), as well as other carbon transfer reactions (see Blakeley, 1969). Treatment with this drug in effect creates a situation of thymidylate starvation or partial depletion. Recent findings of Makino and Munakata (1978) indicate that dUTP is incorporated into DNA under conditions of thymine starvation in thymine requiring strains of *Bacillus subtilis*. It was also found that strains defective in uracil N-glycosylase showed higher survival during the course of thymineless death. So the sensitivity of *rad18-2* strains to trimethoprim could be explained as a defect in excision of dUMP incorporated into DNA during depletion of TMP. Wild type cells, which are resistant to trimethoprim, could remove uracil in DNA without dire consequences.

Additional support for this hypothesis is the result of Prakash (1976) that *rad18* diploids show an increase in reversion caused by nitrous acid (HNO_2), as well as being sensitive to it. One of the chemical lesions caused by HNO_2 is the deamination of cytosine to uracil. In *E. coli* mutants defective in uracil DNA glycosylase (*ung*$^-$) are sensitive to nitrous acid but not MMS (Da Roza *et al.*, 1977). This implicates *RAD18* in removal of uracil from DNA. The increase in mutation cannot be explained by the uracil remaining in the DNA and pairing with adenine at replication giving a GC to AT transition, since this will not explain trimethoprim sensitivity.

An alternative hypothesis which takes into account the possible increase in strand breakage seen in *rad 18-2* is as follows. When base excision repair acts upon uracil in the DNA, the gaps formed will not be filled by strains defective in *RAD18*. These gaps may then be substrate

for mutagenic repair. The fact that *rad6* decreases reversion with HNO_2 (Prakash, 1976) and *rad6* strains cannot repair double-strand breaks after treatment with MMS (Chlebowicz and Jachymczyk, 1979) implicates double-strand breaks. Another implication of this possibility is that there are two modes of repair of double-strand breaks, one mutagenic *rad6* dependent and the other nonmutagenic *rad52* dependent. Prakash (1976) found *rad52* does not change reversion by nitrous acid but was sensitive to HNO_2 compared to the wild type.

Laura DiCaprio has recently found that *rad18* strains in an excision-defective background do not repair discontinuities in DNA replicated on a U.V. induced dimer containing template (pers. comm.). This result is consistent with the findings of this study. The breaks in DNA are found after repair of existing breaks and may coincide with the onset of DNA synthesis. This, however, has not been tested.

One point that may need further verification is whether apurinic sites are being detected on the alkaline sucrose gradients employed in this study. Lindahl and Anderson (1972) reported that alkaline sucrose centrifugation of DNA containing AP sites resulted in little change from control DNA. To detect apurinic sites a pre-incubation with 1 M glycine, 1 M NaOH (pH 12.8) at 25°C for 30 minutes allowed detection of AP sites as breaks on alkaline gradients. So the conditions used of 0.3 M NaOH at 5°C would probably not detect a large majority of AP sites. The breaks seen are then primary breaks induced by γ-rays.

Boram and Roman (1976) have found that *rad18-3* strains have a higher rate of spontaneous mitotic gene conversion. If the results in the present study can be applied to the spontaneous level, the high conversion rate can be explained. If, by acting on spontaneous lesions, the *rad18* defective enzyme leaves gaps in DNA, as in induced lesions

some double-strand breaks are expected to form. This is then a substrate for repair by the *RAD52* epistasis group. It is known that *rad52* and *rad51* strains are defective in induced gene conversion (Resnick, 1975; Morrison, 1978) and in double-strand break repair (Resnick and Martin, 1976; Ho and Mortimer, 1975; and this study). Hence the increase in double-strand breaks will leave substrates for a recombination repair pathway. This should not be taken that single-strand breaks are not substrates for recombination since single-strand invasion is a possible step in recombination (Holloman and Radding, 1976). The above mechanism may aid in explaining some of the increased spontaneous mitotic segregations seen in MMS-sensitive mutants in yeast (Prakash and Prakash, 1977b).

Another anomaly with increased spontaneous mitotic segregation was found in *mut5* diploids by Morrison (1978). He found an increase in the frequency of recessive markers being uncovered on both arms of chromosome V in both heterozygous and homozygous *mut5* diploids compared to wild type cells. It is not known if this was due to two mitotic exchanges on either side of the centromere or the loss of a chromosome by nondisjunction and return to disomy. The finding that *mut5* is defective in double-strand break repair may explain the loss of a chromosome or a piece of a chromosome acting on a spontaneous break in DNA. Resnick and Martin (1976) suggested that during faulty repair of a double-strand break by recombination the homologous chromosome may become altered.

From the foregoing discussion it is now possible to give a possible overall scheme for repair in yeast. The fundamental structure is based upon the three epistatic groups (or pathways) proposed by Brendel and Haynes (1973), Game and Cox (1973), Cox and Game (1974).

The first major pathway is excision repair of pyrimidine dimers or nitrogen mustard damage from DNA (Prakash 1975, 1977a,b; Waters and Moustacchi, 1974; Unrau *et al.*, 1971). Members of the *RAD3* pathway also function in repair of crosslinking. For example, *rad3* is needed for the rejoining of strand breaks caused in the repair of crosslinks induced by trimethyl-psoralen and 365 nm light (Jachymczyk, pers. comm.). The excision mode of repair leaves one template strand intact and is therefore largely non-mutagenic (Lawrence and Christensen, 1976; Prakash, 1976).

The next major group is the *RAD18* pathway which appears to be involved with base excision where an apurinic site is an intermediate step in the repair, as well as being responsible for repair of gaps produced by replication on a damaged template. Mutant genes involved with this pathway are generally X-ray, MMS and U.V. sensitive (Game and Mortimer, 1974; Lawrence and Christensen, 1976; Brendel and Haynes, 1973). This process is largely mutagenic (Lemontt, 1972; Lawrence and Christensen, 1976, 1978; Prakash, 1974, 1976). This pathway may be involved partially with U.V. dimer removal, since dimers persist for a longer time in *rad18-3* strains than wild types as measured by photo-reactivation exposures after dark-holding (Boram and Roman, 1976).

This pathway appears to be primarily involved with removal of damaged bases, misincorporation of bases, for example uracil, and repair of apurinic sites in DNA. If discontinuities are not repaired before the onset of DNA replication double-strand breaks will result. Karren *et al.* (1977) have found single-strand discontinuities in DNA from cells treated with MMS, as detected by the S1 nuclease, showing that the gap filling step is rate limiting after the apurinic site has been cut, at least in human cells. This was not found for excision

type repair as in acetoxyacetylaminofluorene (AAAF) damage.

The *RAD52* pathway seems to be primarily involved in the repair of double-strand breaks or at times when the template strand is not functional as such.(Resnick and Martin, 1976; Ho and Mortimer, 1975; Chelebowicz and Jachymczyk, 1979; this study). This repair appears to be mediated through a recombinational process (Resnick, 1975; Cox and Game, 1974; Morrison, 1978). Double-strand breaks used as substrate for this pathway include those primarily induced as in X-rays; close single-strand breaks or lesions as in high doses of MMS; overlapping regions of excision as in U.V.; breaks caused by replication discontinuities over a damaged template and double strand breaks occurring during cross-link removal. So the *RAD52* group will manifest itself when primary repair systems create situations where the template strand is missing or damaged. Correct information is regained through recombination with a homologue or sister chromatid and is largely non-mutagenic (Prakash, 1976; Lawrence and Christensen, 1976; McKee and Lawrence, 1978).

One other pathway that may exist for which no known mutants exist is repair of primary single-strand breaks after X-rays. The fact that both *rad51-1* and *rad18-2* strains repair single-strand breaks indicates the two primary X-ray repair pathways are not involved in single-strand break repair. An alternative is that single-strand break repair occurs after the *rad18* step in the same pathway.

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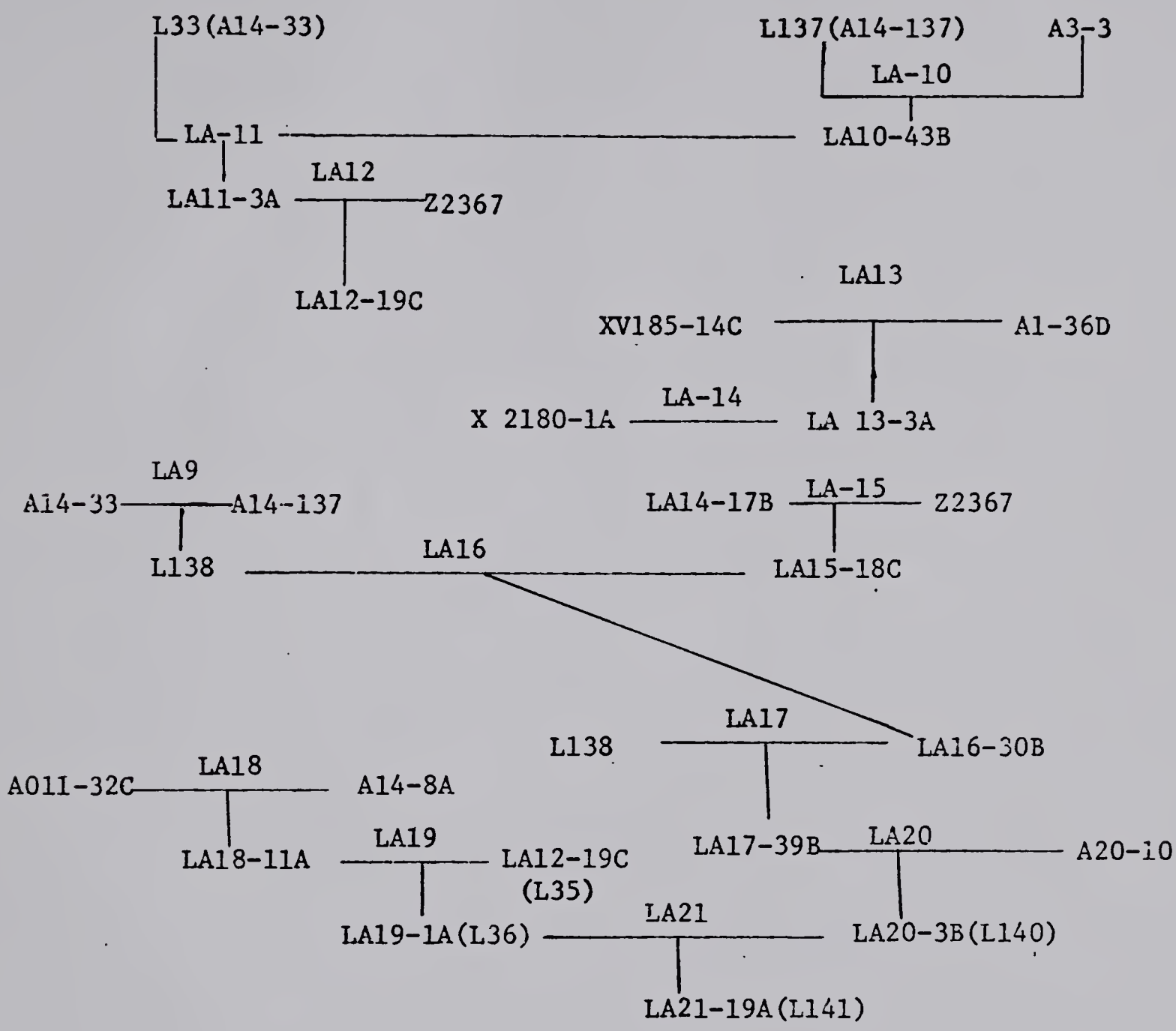
Appendix 1

Pedigree of Strains Used in this Study

a)



b)